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DESARROLLO DE SISTEMAS DE ALERTA Y DE MÉTODOS DE IDENTIFICACIÓN Y DETECCIÓN DE TOXINAS MARINAS

Tesis doctoral

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Abreviaturas y acrónimos

AOAC:	Asociación Oficial de Químicos Analíticos.
ARfD:	dosis de referencia aguda.
ASP:	intoxicación amnésica por consumo de molusco.
AZAs:	azaspirácidos.
BTXs:	brevetoxinas.
C-CTXs:	ciguatoxinas del Caribe.
CDP:	polímero diseñado computacionalmente.
CFP:	intoxicación ciguatérica por consumo de pescado.
CID:	disociación inducida por colisión.
CM:	carne de molusco.
CTXs:	ciguatoxinas.
DA:	ácido domoico.
dcGTXs:	decarbamoilgonyautoxinas.
dcneoSTX:	decarbamoilneosaxitoxina.
dcSTX:	decarbamoilsaxitoxina.
DSP:	intoxicación diarreica por consumo de molusco.
DTXs:	dinofisistoxinas.
EFSA:	Autoridad Europea de Seguridad Alimentaria.
EGMP:	fosfato de metacrilato de etilenglicol.
ELISA:	ensayo de inmunoabsorción ligado a enzimas.
EMS:	espectro de masas aumentado.
EPI:	escaneo de iones producto aumentado.
eq:	equivalentes.
ESI:	ionización por electrospray.
FAO:	Organización de las Naciones Unidas para la Alimentación y la Agricultura.
g:	gramo.
GTXs:	gonyautoxinas.
GYMs:	gimnodiminas.
HABs:	floraciones algales nocivas.
HPLC:	cromatografía líquida de alta eficacia.
HPLC-FLD:	HPLC con detección fluorescente.

HPLC-UV:	HPLC con detección ultravioleta.
I-CTXs:	ciguatoxinas del Océano Índico.
i.p.:	intraperitoneal.
kg:	kilogramo.
L:	litro.
LC-MS:	cromatografía líquida acoplada a espectrometría de masas.
MBA:	bioensayo en ratón.
mg:	miligramo.
mL:	mililitro.
MS/MS:	cromatografía de masas en tándem.
MU:	unidades de ratón.
neoSTX:	neosaxitoxina.
ng:	nanogramo.
NSP:	intoxicación neurotóxica por consumo de molusco.
OA:	ácido okadaico.
PC:	peso corporal.
P-CTXs:	ciguatoxinas del Pacífico.
PlTX:	palitoxina.
PnTXs:	pinnatoxinas.
PP1:	fosfatasa proteica 1.
PP2A:	fosfatasa proteica 2A.
PSP:	intoxicación paralizante por consumo de molusco.
PSTs:	toxinas paralizantes de molusco.
PtTXs:	pteriatoxinas.
PTXs:	pectenotoxinas.
SPATT:	seguimiento de la toxina mediante adsorción en fase sólida.
SPXs:	espirólidos.
SPR:	resonancia de plasmón superficial.
STX:	saxitoxina.
TTX:	tetrodotoxina.
UE:	Unión Europea.
UPLC:	cromatografía líquida de ultra eficacia.
YTXs:	yesotoxinas.

µg:	microgramo.
ºC:	grado centrígrado.

Índice

1. Introducción

En los últimos años, las aguas costeras de muchos países han experimentado una tendencia creciente y preocupante en la incidencia de floraciones de algas nocivas, en inglés <<Harmful Algal Blooms (HABs)>>. El término HAB es muy amplio y abarca floraciones de muchos tipos, aunque todas tienen una característica en común y es que, causan daño, ya sea por la producción de toxinas o por la biomasa acumulada que afecta a los organismos que coexisten alterando la dinámica de la cadena alimentaria. Las HABs se caracterizan por la proliferación de determinadas especies de microalgas tóxicas o nocivas que provocan a menudo discoloraciones en la superficie del mar. Estas algas pueden causar intoxicaciones y muertes de seres humanos, mamíferos marinos, aves, peces y otras formas de vida oceánica, debido a que producen toxinas que son transferidas en la cadena alimentaria.

En la mayor parte de los casos, las HABs están formadas por microalgas inocuas y no constituyen ningún peligro para el ecosistema si se dan en zonas abiertas con buena tasa de renovación del agua. No obstante, éstas pueden resultar perjudiciales si se forman en bahías y ensenadas con escasa circulación.

Como consecuencia de las HABs, el número de toxinas y especies tóxicas así como las pérdidas económicas en el sector pesquero, en el productor de moluscos bivalvos y en la industria transformadora han aumentado en las últimas décadas. Los factores que más han influido en la extensión de este problema han sido el calentamiento global y el efecto antropogénico. La intensidad de las actividades humanas, que se llevan a cabo en las zonas costeras ha producido importantes modificaciones en la estructura de la línea de costa y un deterioro de la calidad de sus aguas. Además, el enriquecimiento de nutrientes relacionado con la contaminación (eutrofización) y la introducción de especies foráneas, ya sea mediante mecanismos naturales de dispersión o a través de las aguas de lastre de los barcos, ha contribuido a incrementar este problema. La diversidad de especies de algas nocivas y sus efectos presentan por lo tanto un reto significativo para los responsables que gestionan los recursos costeros, sin que por el momento se puedan predecir ni puedan ser evitadas.

Por lo tanto, el desarrollo de métodos de identificación y detección de estos productos tóxicos es un objetivo fundamental de la comunidad científica internacional.

1.1. Toxinas marinas

Las toxinas marinas se definen como los productos secundarios, con actividad tóxica originados por el metabolismo de algunas especies de microalgas fitoplanctónicas (dinoflagelados, diatomeas y cianobacterias). De las 5000 especies de microalgas existentes, cerca de 2000 dinoflagelados, existen unas 100 especies capaces de producir poderosas toxinas en su metabolismo. Estas toxinas son compuestos bioactivos de distinta naturaleza química que pueden presentar efectos hemolíticos, neurotóxicos o enterotóxicos dependiendo de su estructura, estado de conversión, dosis y susceptibilidad del consumidor, sea éste humano o animal. Dentro de las diatomeas marinas solamente las del género *Pseudo-nitzcshia* han sido relacionadas con la producción de toxinas [1, 2]. En cuanto a las cianobacterias, se estima que más del 50% de las floraciones de cianobacterias de aguas continentales, son tóxicas [3]. Si bien otras bacterias están además implicadas en la producción de neurotoxinas que afectan gravemente a la salud humana [4, 5].

Históricamente la clasificación de las toxinas marinas ha sido abordada desde diferentes perspectivas, en particular: similitud estructural, distribución taxonómica y filogenética, propiedades químicas (polaridad, carácter lipofílico…), en base a su modo de acción o de acuerdo con los síntomas observados en humanos [5-7]. Pero con el tiempo, la aparición de nuevas toxinas y los avances en los estudios farmacológicos hicieron insuficiente esta clasificación. Desde el 2004, expertos de la Organización de las Naciones Unidas para la Alimentación y la Agricultura (FAO), recomiendan la clasificación de las toxinas marinas en función de su estructura química [8].

1.1.1. Clasificación de las toxinas marinas

En base a la clasificación de la FAO se pueden distinguir los siguientes grupos de toxinas:

- grupo de la saxitoxina y sus derivados, que inducen la intoxicación paralizante por consumo de molusco (paralytic shellfish poisoning, PSP).
- grupo del ácido domoico y sus derivados, causantes de la intoxicación amnésica por consumo de molusco (amnesic shellfish poisoning, ASP).
- grupo de la tetrodotoxina y sus derivados.
- grupo de las ciguatoxinas, responsables de la intoxicación ciguatérica por consumo de pescado (ciguateric fish poisoning, CFP).
- grupo de las palitoxinas.
- grupo del ácido okadaico y sus derivados, que inducen la intoxicación diarreica por consumo de molusco (diarrheic shellfish poisoning, DSP).
- grupo de las pectenotoxinas.
- grupo de las yesotoxinas.
- grupo de los azaspirácidos.
- grupo de las brevetoxinas, que causan la intoxicación neurotóxica por consumo de molusco (neurotoxic shellfish poisoning, NSP).
- grupo de las iminas cíclicas. (Ejemplo: gimnodiminas y espirólidos).

- **Grupo del ácido domoico**

El ácido domoico (DA) es un compuesto natural perteneciente a la categoría de los kainoides aislados de diversas fuentes marinas, incluidas macro y micro algas. El DA se identificó por primera vez como la toxina responsable del síndrome ASP que ocurrió en Canadá en 1987 tras el consumo de mejillones contaminados (*Mytilus edulis*) [9]. La causa del DA en estos mejillones fue una proliferación de las microalgas del género *pseudo-nitzschia* [10]. Desde entonces, el DA ha provocado la mortalidad de cientos de aves marinas [11, 12], mamíferos y peces [13-15]. El DA también ha sido detectado en varias especies de moluscos en todo el mundo, incluyendo Estados Unidos, Nueva

Zelanda, México y varios países europeos como Francia, Portugal, Irlanda y España [16-19]. Originalmente, fue descubierto como un producto del alga roja, *Chondria armata,* y aislado posteriormente de otras microalgas rojas y varias especies de diatomeas, principalmente del género *Pseudo-nitzschia* (*multiseries*, *pseudodelicatissima*, *australis*, *seriata,*…) [10, 20-22]. Aunque el DA era la toxina mayoritaria, otros análogos han sido identificados en estos organismos así como en los tejidos de otros moluscos (berberechos, almejas, mejillones, ostras o navajas) [18, 23].

El compuesto original es un amino ácido tricarboxílico, termoestable e hidrosoluble cuya estructura química, que se presenta en la figura 1, es muy similar a la de los aminoácidos neuroexcitadores: ácido glutámico y ácido kaínico [24].

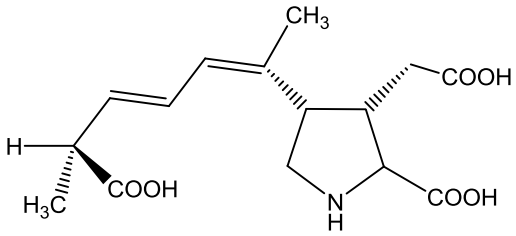


Figura 1: Estructura del ácido domoico.

El mecanismo de acción del DA se caracteriza por la activación de los receptores de glutamato en el sistema nervioso central. El DA tiene una alta afinidad por el ácido alfa-amino-3-hidroxi-5-metil-4-isoxazolpropiónico y las subunidades de los receptores de kainato que están presentes en el sistema nervioso central de los mamíferos [25]. La interacción de DA y estos receptores de glutamato provoca la despolarización de la célula y como consecuencia la disfunción y la muerte celular [26, 27]. Los síntomas clínicos de la intoxicación ASP incluyen trastornos gastrointestinales, fuertes dolores de cabeza, pérdida de la memoria, pérdida de equilibrio, alteraciones de la visión, desorientación, convulsiones, agitación, coma y muerte [28, 29].

En la Unión Europea (UE), así como en Canadá y los Estados Unidos el límite reglamentario para el DA es de 20 mg DA/kg en los tejidos comestibles. Varias técnicas analíticas han sido desarrolladas para la detección del DA y sus

análogos en muestras de agua, algas y moluscos. La cromatografía líquida de alta eficacia (HPLC) con detección ultravioleta (HPLC-UV) fue el primer método analítico para la determinación del DA [30-32] y todavía es el método más empleado en la actualidad. De hecho ha sido validado y estandarizado como método de referencia para la cuantificación del DA [33, 34]. Además, el método basado en el ensayo de inmunoabsorción ligado a enzimas (ELISA) también ha sido validado [35] y se utiliza oficialmente en la UE para fines de cribado. Estos métodos tienen límites de detección lo suficientemente bajos como para detectar el DA a una concentración de 4,5 mg/kg de carne de molusco (CM). Esta dosis se estableció como la cantidad máxima que debe contener una porción de 400 g de CM para no superar la dosis de referencia aguda (ARfD) de 30 µg DA/kg peso corporal (PC) [36]. Además, la técnica de cromatografía líquida con detección por espectrometría de masas (LC-MS), puede ser una herramienta valiosa para la determinación rápida y selectiva del DA y sus isómeros en extractos crudos de molusco [37]. Se han desarrollado otros métodos químicos tales como biosensores basados en la resonancia de plasmón superficial (SPR), electroforesis capilar [38] y ensayos en cultivos neuronales [24]. Pero estos métodos se utilizan en raras ocasiones y todavía no han sido validados en estudios interlaboratorios [36].

- **Grupo de las ciguatoxinas**

Las ciguatoxinas (CTXs) son productos del metabolismo de las toxinas producidas por los dinoflagelados bentónicos del género *Gambierdiscus toxicus* [39]. Los peces herbívoros concentran las toxinas cuando se alimentan de algas asociadas con *Gambierdiscus* y posteriormente son transferidas, transformadas y acumuladas en el hígado, músculo, piel y espinas de los peces carnívoros de gran tamaño llegando así a los niveles más altos de la cadena trófica. *G. toxicus* es también la fuente de otros tipos de toxinas marinas como las maitotoxinas y el gambierol [39].

Estructuralmente, las CTXs son compuestos lipídicos poliéteres solubles y relativamente estables al calor, formados por 13 y 14 anillos unidos por enlaces éter con una estructura muy rígida del tipo escalera (figura 2) [40]. Son un grupo de más de 20 análogos, de los cuales 12 han sido caracterizado estructuralmente y se clasifican en tres grupos según su distribución

geográfica: CTXs del Caribe (C-CTXs), del Pacífico (P-CTXs) y del Océano Índico (I-CTXs) [41].

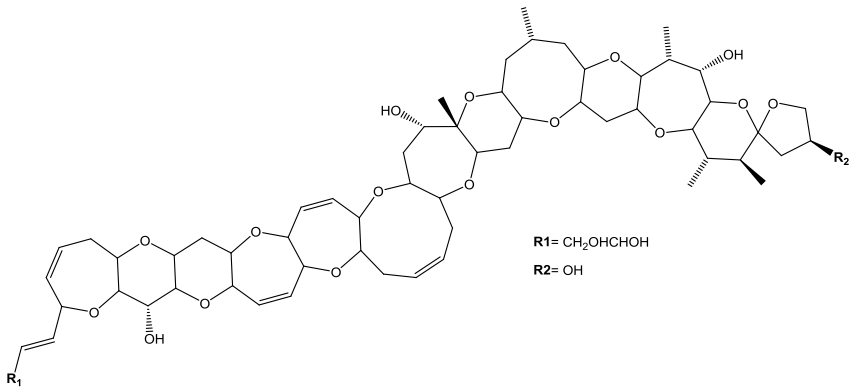


Figura 2: Estructura de la ciguatoxina (P-CTX-1).

Las CTXs son consideradas como la causa principal de la intoxicación CFP en los seres humanos. La CFP es endémica de las regiones tropicales y subtropicales con arrecifes coralinos, sin embargo su incidencia está aumentando en otras partes del mundo debido a la expansión y desarrollo del comercio internacional de varias especies de peces tropicales [42]. Recientemente estas toxinas han sido detectadas en el Mediterráneo [43, 44] y en el Atlántico, a la altura del paralelo 28 de latitud Norte [45, 46].

Las CTXs se caracterizan por su unión con alta afinidad al sitio 5 de los canales de sodio dependientes de voltaje. Esto resulta en un aumento intracelular de iones Na^{+} y también de iones Ca^{+2}, una despolarización de la célula y en la aparición de potenciales de acción espontáneos en las células excitables [47, 48]. Los síntomas de la intoxicación se caracterizan por gastroenteritis aguda (vómitos, diarrea, náuseas y dolor abdominal), seguido de trastornos neurológicos, alteraciones cardiovasculares, síntomas músculo-esqueléticos y disestesia [49].

Actualmente no hay límites reglamentarios para las CTXs en la UE. Sin embargo de acuerdo a la legislación vigente, los productos pesqueros que contengan CTX o tetrodotoxina no deben ser colocados en el mercado [50]. Así mismo, la Autoridad Europea de Seguridad Alimentaria (EFSA) no ha podido establecer una ARfD para estas toxinas debido a la limitada información de esta clase de toxinas marinas [51].

En los últimos años se han desarrollado métodos para determinar la presencia de las CTXs y su distribución. Dentro de los métodos biológicos más usados y tradicionales está el bioensayo en ratón (MBA) a pesar de su falta de especificidad [52]. Más recientemente se han utilizado anticuerpos policlonales y monoclonales para ELISA [53], además de ensayos celulares o de citotoxicidad [40, 54], y la evaluación cuantitativa de la actividad biológica mediante la utilización del patch-clamp [55]. También se han desarrollado técnicas analíticas basadas en la LC acoplada a espectrometría de masas en tándem (MS/MS) y cromatografía líquida de ultra eficacia (UPLC) para la determinación de CTXs en extractos de pescado [46, 56, 57].

- **Grupo de las palitoxinas**

La palitoxina (PlTX) es una de las toxinas no proteicas más potentes conocidas hasta la fecha. Fue originalmente aislada del zoántido *palythoa toxica* en los arrecifes coralinos de las islas Hawaianas [58]. Posteriormente, la PlTX y varios de sus análogos fueron detectados en diferentes especies tropicales de *Palythoa* [59-61] y dinoflagelados bentónicos del género *Ostreopsis* (*O. ovata*, *O. siamensis*, *O. mascarenensis*) [62-64]. Aunque se describieron por primera vez en Hawai y Japón, estas toxinas están actualmente distribuidas por todo el mundo. En la última década se ha informado de floraciones de *Ostreopsis* en cuatro países europeos: Francia, Grecia [65-67], Italia [68] y España [69, 70].

La estructura química de la PlTX, que se presenta en la figura 3, posee una larga cadena de alquilo, parcialmente insaturada (con ocho dobles enlaces), 7 anillos éter, 64 centros quirales, 40-42 grupos hidroxilo y dos grupos amida [59, 71].

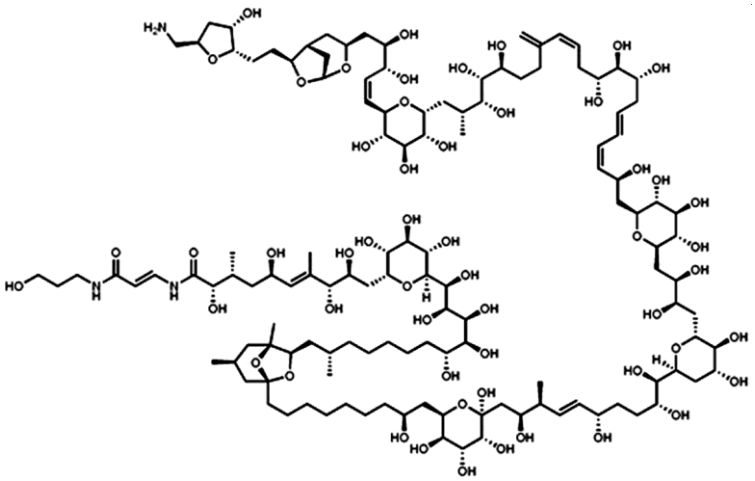


Figura 3: estructura química de la palitoxina.

La distribución, toxicología, modo de acción, y métodos de detección de la PlTX se han revisado recientemente [67, 72-74]. La principal diana biológica de la PlTX parece ser la bomba ATPasa Na^+ / K^+ de la membrana plasmática, que participa en el mantenimiento de los gradientes iónicos de trans-membrana de las células animales y que es esencial en numerosas funciones celulares [75-77]. La unión de la PlTX inhibe el funcionamiento de la ATPasa y produce la formación de un poro, permeable a cationes monovalentes, destruyendo así el gradiente iónico en la membrana citoplasmática. Estas alteraciones de la membrana celular desencadenan una serie de señales intracelulares que pueden dar lugar a contracciones del músculo liso y esquelético y varios efectos biológicos adversos [78]. Las PlTXs representan un riesgo para el ser humano por diferentes rutas: ingestión, inhalación y dérmica [79, 80]. Los síntomas de la intoxicación incluyen vasoconstricción, hemorragia, ataxia, debilidad muscular, fibrilación ventricular, hipertensión pulmonar, isquemia, y la muerte [67].

En la actualidad, no existe una normativa para el control de las PlTXs en pescados y mariscos, pero la EFSA ha propuesto una ARfD de 0,2 µg PlTX/kg PC y ha recomendado que para no exceder esta dosis, una porción diaria de 400 g de CM no debe contener más de 30 µg de la suma de PlTXs por kg de carne [81].

Se han utilizado numerosos ensayos y análisis para detectar PlTXs entre los que se incluyen, inmunoensayos [82-84], ensayos de neutralización para la actividad hemolítica [85], métodos químicos basados en la espectroscopia infrarroja, ultravioleta, MS, electroforesis capilar, LC o biosensores SPR [73, 86, 87]. Aunque no existe un método oficial de detección, ni como se ha comentado una normativa oficial en la UE para su control.

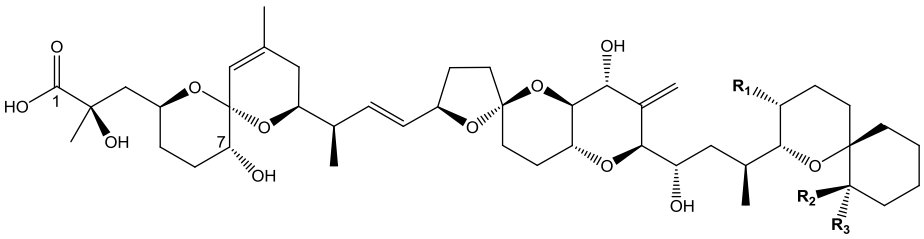
- **Grupo del ácido okadaico**

El síndrome DSP es causado por un grupo de compuestos poliéteres liposolubles que incluyen, el ácido okadaico (OA) y varios de sus análogos estructurales, las dinofisistoxinas (DTXs), DTX-1 y DTX-2 así como un grupo de formas esterificadas de estas toxinas.

Los primeros episodios de DSP tuvieron lugar en Holanda en la década de los 60 [88], seguidamente se registraron a finales de los años 70 en Japón, donde ocasionaron grandes problemas en el cultivo de vieiras [89]. Se señaló al dinoflagelado *Dinophysis fortii* como el causante de estos episodios [90]. Desde entonces, más de 1300 casos han sido registrados en este mismo país entre 1976 y 1982, en España más de 5000 casos en 1981 y aproximadamente 3300 en Francia en 1983. En 1984, la industria del mejillón permaneció cerrada prácticamente todo el año en Suecia por las toxinas DSP. Actualmente, estas toxinas están ampliamente distribuidas en todo el mundo siendo especialmente importantes en Europa y Japón, donde aparecen de manera estacional [91-93].

El OA debe su nombre a la esponja marina de donde se aisló originalmente, *Halichondria okadaii* [94], mientras que las DTXs lo deben a uno de los géneros de dinoflagelados que las producen, *Dinophysis* (*D. fortii*, *D. acuminata*, *D. acuta*, *D. norvegica*, *D. mitra*, *D. rotundata*, *D. tripos*, *D. caudata*, *D. sacculus*) [95, 96]. También pueden ser producidas por dinoflagelados del género *Prorocentrum* (*P. lima*, *P. concavum* o *P. maculosum*, *P. redfieldi*, *P. Arenarium* y *P. belizeanum*) [97, 98]. Las toxinas DSP se acumulan en los moluscos filtradores, especialmente mejillones y ostras, aunque también pueden encontrarse en almejas, vieiras, navajas y cangrejos [99, 100]. Dado que son sustancias lipofílicas tienden a ser más abundantes en las partes grasas del animal.

La identificación estructural del OA y la DTX-1 se esclareció por primera vez en 1982 [101], seguidamente la DTX-2 fue aislada a partir de mejillones tóxicos en 1992. Estas toxinas están formadas por una cadena de 38 carbonos con un grupo carboxilo en el C-1 y un grupo hidroxilo en el C-7 y se diferencian entre sí en el número o la posición de grupos metilo (Figura 4). Estos compuestos pueden ser acilados con ácidos grasos, saturados o insaturados a través de la esterificación del grupo hidroxilo en el C-7 produciendo una mezcla de ésteres de 7-O-acilo globalmente designados como DTX-3 [102].



	R_1	R_2	R_3
OA	CH_3	H	H
DTX-1	CH_3	CH_3	H
DTX-2	H	H	CH_3
DTX-3	CH3	CH_3	acilo

Figura 4: Estructura de las principales toxinas del grupo del ácido okadaico.

En cuanto a su mecanismo de acción, el OA, la DTX-1 y DTX-2 son potentes inhibidores de las fosfatasas de proteína con residuos de serina/treonina, especialmente de la PP1 y PP2A [103, 104]. Este mecanismo de acción es probablemente el responsable de la inflamación del tracto intestinal, diarreas y efecto promotor de tumores [105, 106]. Estas fosfatas están involucradas en múltiples procesos de regulación celular, tales como el metabolismo, el transporte y la secreción en la membrana, la contractilidad y la división celular. Algunos autores señalan que la causa de la diarrea en el ser humano es la hiperfosforilación de las proteínas que controlan la secreción de sodio de las células intestinales o a un aumento de la fosforilación de las proteínas citoesqueléticas o de unión, responsables de regular la permeabilidad de los solutos, lo que ocasiona una pérdida de fluidos [107, 108]. Los principales síntomas de la intoxicación derivan, por lo tanto en trastornos puramente gastrointestinales, como náuseas, vómitos, diarrea y dolor abdominal, que

aparecen entre 30 minutos y unas horas después de la ingestión de los moluscos contaminados. La recuperación completa se observa después de tres días. Actualmente todavía no se ha registrado ningún caso de intoxicación mortal en humanos, ni tampoco en aves, peces ni mamíferos marinos.

El análisis de este grupo de toxinas representa un aspecto clave en los programas de monitoreo de todo el mundo. El MBA se estableció como método oficial de detección de las toxinas DSP, sin embargo este método no se considera el más adecuado ya que puede dar lugar a resultados cruzados con otras toxinas lipofílicas apareciendo falsos positivos en la detección.

Actualmente en la UE el límite máximo permitido para el OA y las DTXs (incluidas pectenotoxinas) es de 160 µg OA equivalentes (eq)/kg de CM [109]. Además, la EFSA indica que una porción de 400 g de CM no debe contener más de 45 µg de OA eq/kg CM para así no exceder la ARfD (0,3 µg OA eq/kg PC) [110].

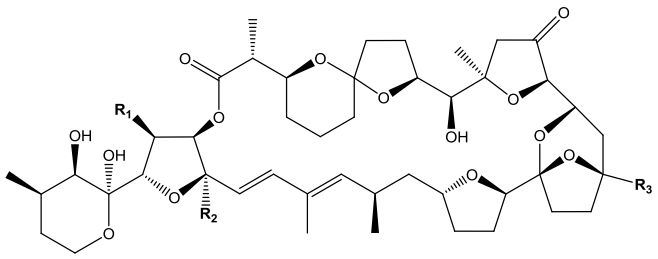
Para detectar estas toxinas la regulación EC Nº 2074/2005 establece que se pueden usar distintos tipos de bioensayos que difieren en la porción analizada (hepatopáncreas o cuerpo entero) y en los disolventes empleados en el proceso de extracción y purificación. Además se pueden utilizar métodos de detección alternativos como la HPLC con detección fluorescente (HPLC-FLD), el LC-MS, inmunoensayos y ensayos de inhibición de fosfatasas, sólo si demuestran que son tan efectivos como los métodos biológicos [111]. Recientemente la técnica del LC-MS ha sido validada por los laboratorios de referencia de toxinas de la UE en un estudio de validación interlaboratorio [112] para la detección de toxinas lipofílicas. Se han realizado varios estudios para comprobar la exactitud, precisión y la recuperación de este método [113, 114]. Sin embargo, se ha visto que el método necesita definirse mejor para obtener cuantificaciones adecuadas sin errores y así asegurar la protección de los consumidores [115].

- **Grupo de las pectenotoxinas.**

Las pectenotoxinas (PTXs) son una familia de poliéteres macrocíclicos que contaminan moluscos en muchas partes del mundo, incluyendo, Australia [116], Croacia [117, 118], Irlanda [119], Italia [120], Japón [95, 121], Nueva Zelanda [122], Noruega [95, 123], Portugal [99, 124], España [125], Rusia [126] y Reino

Unido [127]. Aparecen a menudo con las toxinas del grupo del OA ya que son producidas por el mismo género de dinoflagelados, *Dinophysis*. El primer dinoflagelado identificado como productor fue *D. fortii* [128] pero posteriormente las PTXs se encontraron en *D. acuminata*, *D. norvegica*, *D. rotundata*, *D. acuta* y *D. caudata* [95, 125, 129, 130]. Se detectaron en moluscos por la alta toxicidad aguda mostrada en el MBA después de la inyección intraperitoneal (i.p.) de extractos lipofílicos [131, 132]. Debido a la asociación con el OA y las DTXs, las PTXs han sido tradicionalmente incluidas en el grupo de las toxinas DSP, sin embargo varios estudios en animales indican que las PTXs son mucho menos tóxicas por vía oral y no inducen diarrea. Por esta razón y porque tienen una estructura química diferente a la del OA, las PTXs han sido clasificadas como un grupo independiente. A pesar de su aparente falta de toxicidad oral, las PTXs son tóxicas en ratones por vía i.p. [133] y por ello son una posible fuente de falsos positivos en la detección de toxinas DSP cuando se utiliza el MBA.

Estructuralmente se parecen al OA en el peso molecular, en que tienen éteres cíclicos y un grupo carboxílico en la molécula, sin embargo se diferencian en que el grupo carboxílico está en una forma de lactona macrocíclica (macrólido) [134] (Figura 5). La PTX-1 y PTX-2, principales pectenotoxinas del grupo, fueron inicialmente aisladas de las vieiras japonesas *Patinopecten yessoensis* [131, 135]. Hasta la fecha se han aislado y caracterizado quince compuestos distintos. A diferencia de la mayoría de los análogos, que han sido aislados de varias especies de *Dinophysis*, la PTX-1, PTX-3 y PTX-6 no están presentes en las microalgas sino que son formadas por el metabolismo de la PTX-2 en los tejidos de los moluscos. Por lo tanto, estas toxinas son el resultado de conversiones enzimáticas en donde el grupo metil en el C18 de la molécula de la PTX-2 es progresivamente oxidado a un alcohol (PTX-1), a un aldehído (PTX-3) y finalmente a un ácido carboxílico (PTX-6) [133, 136].



	R_1	R_2	R_3
PTX1	H	H	CH_2OH
PTX2	H	H	CH_3
PTX3	H	H	CHO
PTX6	H	H	COOH

Figura 5: Estructura de las principales pectenotoxinas.

A pesar de que las toxinas del grupo de las PTXs no comparten el mismo mecanismo de acción que las del grupo del OA (ambas registradas de manera conjunta en la normativa europea), se tienen que incluir en el mismo límite regulador (160 µg OA eq/kg CM) [109] y los métodos de detección son los mismos que para las toxinas del grupo del OA [112]. Basándose en los datos de toxicidad del MBA, la EFSA estable que para no exceder la ARfD (0,8 µg de PTX2 eq/kg PC), una porción de 400 g de CM no debe contener más de 120 µg de PTX2 eq/kg CM [137].

- **Grupo de las yesotoxinas**

Las yesotoxinas (YTXs) representan un grupo de compuestos poliéter liposolubles producidos por los dinoflagelados de las especies *Protoceratium reticulatum*, *Lingulodinium polyedrum* y *Gonyaulax spinifera* [138-140]. El compuesto principal del grupo es la YTX-1, cuya estructura planar se estableció primero y fue la base para la determinación de sus análogos [141]. Su estructura se compone de 11 anillos éter encadenados formando una cadena principal de 47 carbonos, una cadena lateral de 9 carbonos y dos grupos sulfónicos (Figura 5).

La YTX-1 se aisló por primera vez en 1986 en la Bahía de Mutsu, Japón [141] de la glándula digestiva de *Patinopecten yessoensis*, una vieira que dio su nombre a la toxina. Desde entonces, las YTXs han sido identificadas en

moluscos de Noruega [142], Chile, Nueva Zelanda [143], Reino Unido [127], Canadá [144], Rusia [126], Italia [145], España y Estados Unidos [139]. Actualmente se conocen 40 derivados naturales de la YTX que han sido identificados y caracterizados mediante estudios de espectroscopia de resonancia magnética nuclear y/o LC-MS. Algunos son producidos directamente por dinoflagelados, mientras que otros son producidos por el metabolismo de los moluscos. [146, 147].

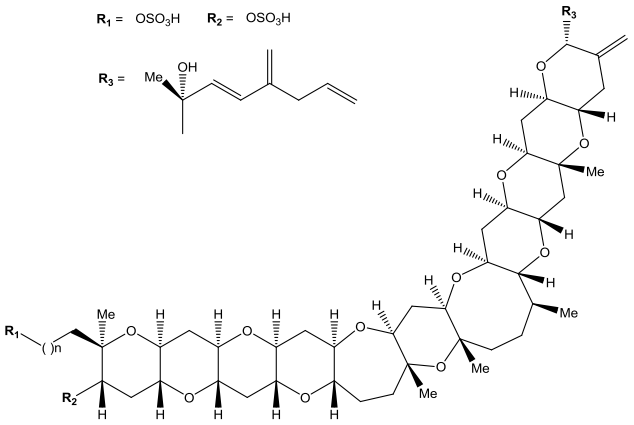


Figura 6: Estructura de la yesotoxina.

Las YTXs fueron originalmente incluidas entre las toxinas responsables de DSP principalmente porque coexisten en muestras de molusco contaminado y son coextraídas en la misma fracción lipofílica que el OA y las DTXs. Sin embargo, las YTXs no son diarreogénicas y comparadas con el OA muestran 4 veces menos potencia en la inhibición de la PP2A [148]. Estudios toxicológicos en ratones revelan que la YTX es más tóxica que las toxinas DSP por vía i.p [107], por el contrario su toxicidad oral es más débil ya que dosis de 1 mg/kg no son letales para los roedores [148-152]. Esta es la principal causa de los falsos positivos en la detección de toxinas DSP en el MBA. Por esta razón se propuso que la YTX y todos sus análogos fueran excluidos del conjunto de las toxinas DSP y pasaran a formar parte de un grupo propio. En cuanto al mecanismo de acción, se ha demostrado que la YTX modula la homeostasis del calcio en linfocitos humanos [153, 154] y disminuye los niveles intracelulares del adenosina monofosfato cíclico a través de la activación de las fosfodiesterasas

celulares en estas mismas células [155-157]. Por otro lado la YTX produce apoptosis en células de neuroblastoma y en células humanas HeLa por activación de caspasas [158-160], e induce la disrupción del sistema E-caterina-catenina en células epiteliales [161].

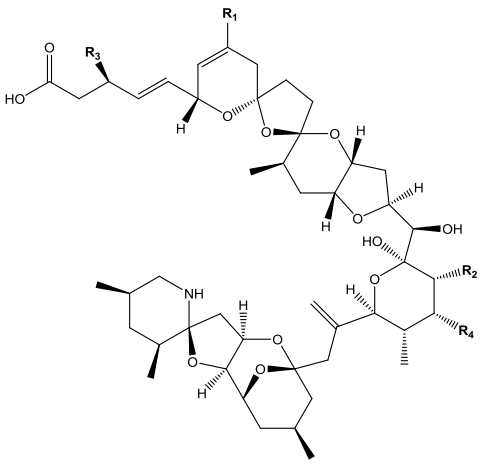
Actualmente, la EFSA establece un límite de control superior para las YTXs de 1 mg de YTX eq/kg [162] y los métodos establecidos para detectarlas son los mismos que para las toxinas del grupo del OA, aunque con limitaciones en el uso de disolventes [112]. Así mismo, la EFSA concluyó que para no exceder la ARfD de 25 µg de YTX eq/kg PC, una porción de 400 g de CM no debe contener más de 3,75 mg YTX eq/kg CM [163].

Este grupo de toxinas también puede ser detectado mediante métodos electroforéticos [164], de fluorescencia de polarización [165], ELISA [166] y biosensores SPR [167, 168], entre otros.

- **Grupo de los azaspirácidos.**

Los azaspirácidos (AZAs) son un importante grupo de biotoxinas lipofílicas que causan intoxicación por consumo de moluscos. El primer episodio tóxico se produjo en Holanda en 1995 tras el consumo de mejillones (*Mytilus edulis*) importados de la costa de Irlanda [169]. Los síntomas de dicha intoxicación fueron similares a los producidos por el síndrome DSP, pero caracterizados, en el MBA, por la aparición de signos de neurotoxicidad con dificultad respiratoria y una lenta parálisis progresiva antes de la muerte [170, 171]. De la carne de estos mejillones se extrajo el AZA1, cuya estructura está formada por un sistema de anillos azaspiro de 5 y 6 miembros, uno de ellos unido a un anillo 2,9-dioxobiciclononano y en el otro extremo de la molécula un ácido carboxílico (Figura 7) [172-174]. A raíz de una segunda intoxicación causada por el consumo de mejillones procedentes de Irlanda que tuvo lugar en 1997 [175], se aislaron y definieron estructuralmente dos análogos del AZA-1, el 8-metilazaspirácido (AZA2) y 22-dimetilazaspirácido (AZA3) [176, 177]. En menor cantidad se identificaron el AZA4 y AZA5, derivados hidroxilados del AZA3, menos frecuentes en la naturaleza [178]. A partir del año 2002, se identificaron 6 nuevos análogos, (AZA6-11). Los AZA4-AZA11 sólo han sido aislados a partir de extractos de marisco, mientras que los AZA1-AZA3 también han sido identificados en los extractos de células de *Protoperidinium crassipes* [179], lo

que sugiere que el resto de los análogos son productos de la bioconversión en los moluscos y eso explicaría la poca abundancia de estos análogos en la naturaleza.



	R_1	R_2	R_3	R_4
AZA1	H	CH_3	H	H
AZA2	CH_3	CH_3	H	H
AZA3	H	H	H	H
AZA4	H	H	OH	H
AZA5	H	H	H	OH
AZA6	CH_3	H	H	H
AZA7	H	CH_3	OH	H
AZA8	H	CH_3	H	OH
AZA9	CH_3	H	OH	H
AZA10	CH_3	H	H	OH
AZA11	CH_3	CH_3	OH	H

Figura 7: Estructura de los azaspirácidos.

Aunque inicialmente los AZAs solo aparecían en mejillones (*Mytilus edulis*) procedentes de Irlanda [180], más tarde se detectaron en otros moluscos bivalvos, como ostras (*Crassostrea gigas, Ostrea edulis*), vieiras (*pecten maximus*), almejas (*Tapes phillipinarium*), berberechos (*Cardium edule*) y también en peces (*Ensis siliqua*) y cangrejos (*Cancer pagurus*) [181, 182]. Del mismo modo, moluscos contaminados con AZAs han sido detectados en áreas como el noroeste de Inglaterra y sureste de Noruega [180], noroeste de Francia y España [183] y noroeste de Marruecos [184].

Existen algunas evidencias de que los AZAs, a diferencia de las toxinas DSP, pueden acumularse no sólo en el hepatopáncreas (glándula digestiva), sino también en las gónadas [183] y en el músculo abductor [185, 186]. Sin embargo, la distribución de una toxina a través de los diferentes órganos en el mismo animal es muy difícil de estudiar debido a problemas de contaminación cruzada entre órganos. Parece ser que el AZA1 es la toxina predominante en las glándulas digestivas y el AZA3 predominante en el resto de los tejidos [181].

En cuanto al mecanismo de acción de estas toxinas, hay varios trabajos publicados que apuntan a que los AZAs modulan los niveles de Ca^{+2} citosólico en linfocitos humanos, tienen efectos en el pH intracelular y además el AZA1

disminuye la viabilidad celular en cultivos neuronales. Estudios *in vitro* también sugieren que el AZA1 afecta al citoesqueleto de actina en las células no adherentes [187-190]. Sin embargo, la diana intracelular de estas toxinas todavía no se ha determinado con certeza.

La UE determinó que el nivel máximo de AZAs (AZA1-AZA3) en los moluscos bivalvos, equinodermos, tunicados y gasterópodos marinos es de 160 µg de AZA1 eq/Kg CM (medido en el cuerpo entero o cualquier parte consumible por separado) [109, 111]. La EFSA estableció una ARfD de 0,2 µg AZA1 eq/kg PC y que para no exceder esta dosis, una porción de 400 g de CM no debe contener más de 30 µg AZA1 eq/kg CM [191]. La detección y cuantificación de estas toxinas se realiza habitualmente mediante las técnicas de LC-MS/MS [113, 192-194].

- **Grupo de las brevetoxinas**

Las brevetoxinas (BTXs) son potentes neurotoxinas producidas por el dinoflagelado *Karenia brevis* (anteriormente conocido como *Gymnodinium breve* y *Ptychodiscus brevis*) [195]. Este dinoflagelado aparece de forma natural en el Golfo de México, Mar Caribe y a lo largo de las costas de Nueva Zelanda y regularmente produce floraciones en las costas de Florida y Texas [196]. Se han identificado otras especies de algas productoras de BTXs, incluyendo *Karenia* y *Chatonella* en todo el mundo [197]. Estos dinoflagelados son los responsables de grandes mortandades de peces y otros animales acuáticos, incluidos mamíferos marinos [198, 199], a través de la exposición directa durante las HABs, o indirectamente en la cadena alimenticia. Muchos moluscos que se alimentan por filtración acumulan BTXs sin efectos adversos evidentes. Sin embargo, la presencia de estas toxinas en sus tejidos representa un riesgo significativo para la salud humana.

El grupo de las BTXs está formado por más de 10 derivados poliéteres liposolubles. Todas estas toxinas derivan de dos esqueletos básicos formados por un anillo lactona y 10 (tipo A) y 11 (tipo B) anillos éter (figura 8) [200, 201]. Dentro del grupo B se incluyen los análogos más abundantes en la naturaleza, las BTX-2 y 3, y las formas 5, 6, 8 y 9. El grupo A se caracteriza por presentar una estructura más flexible lo que determina una mayor potencia de acción, dentro de este grupo se encuentran los análogos 1, 7 y 10 [202].

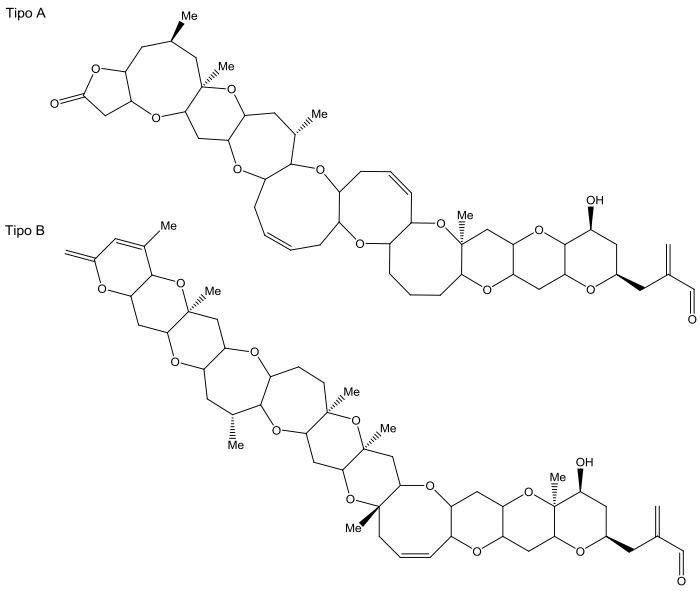


Figura 8: Estructura de las brevetoxinas.

El síndrome NSP se debe principalmente a la despolarización de la célula por acción de las BTXs, a través de la activación de los canales de sodio dependientes de voltaje. De esta forma se alteran las propiedades de membrana de las células excitables favoreciendo el flujo de iones Na^+ hacia el interior de la célula inhibiendo las transmisiones neuronales en el músculo esquelético [203, 204]. Los signos y síntomas de la intoxicación son principalmente gastrointestinales (dolor abdominal, náuseas, vómitos, diarrea) y neurológicos (ataxia, mialgias, parestesias, y reversión de la sensación de temperatura). Estos aparecen en el período de 30 minutos a 3 horas después del consumo de marisco contaminado y duran unos pocos días [205]. Aunque las BTXs se han relacionado con la muerte de peces, aves marinas y algunos mamíferos [206-208], no se han registrado ni síntomas crónicos ni muertes en humanos. Además se han detectado irritaciones de las mucosas por exposición a los aerosoles formados a partir de las floraciones de estos organismos [209, 210].

Hasta la fecha las BTXs no han sido notificadas en moluscos o pescados procedentes de Europa, y actualmente no hay límites reglamentarios para estas toxinas a nivel de la UE. Sin embargo, el descubrimiento de nuevas algas productoras de BTXs y la tendencia expansiva de la distribución de las

floraciones de algas, indican que podrían producirse también en Europa. En la actualidad, el método de detección aceptado para las BTXs es el MBA con el uso de éter dietílico para la extracción de los tejidos de molusco [211]. Básicamente cualquier nivel detectable de BTXs por cada 100 g de carne se considera potencialmente no apto para el consumo humano. En la práctica, la toxicidad de residuos ≥ 20 unidades ratón (MU)/100 g de carne, lo que equivale a 80 µg/mL BTX-2 equivalente, fue adoptado como el nivel de orientación para el cierre de zonas de recolección en Estados Unidos, México y Nueva Zelanda [8, 212].

Como alternativa al MBA se han desarrollado estudios *in vitro* (ELISA) [213], inmunoensayos [214] y ensayos de unión a receptor [215]. Otros métodos han sido desarrollados basados en la SPR [216], inmunosensores [217] y biosensores neuronales [218]. Además se han empleado métodos analíticos para la detección de este grupo de toxinas, como la técnica LC-MS [219, 220] y la cromatografía capilar [221].

- **Grupo de las iminas cíclicas**

Las iminas cíclicas son un grupo heterogéneo de compuestos químicos naturales con características comunes macrocíclicas y la presencia de un grupo funcional imino. Este grupo incluye las gimnodiminas (GYMs) [222], espirólidos (SPXs) [223], pinnatoxinas (PnTXs) [224], pteriatoxinas (PtTXs) [225], prorocentrólidos [226, 227] y espiro-prorocentrimina [228]. A excepción de los SPXs, los cuales parecen tener una distribución global, las otras iminas cíclicas sólo se han encontrado en ciertos lugares y en un grupo taxonómico limitado de especies. Así las PnTXs sólo se han encontrado en los bivalvos *Pinna attenuata* y *Pinna muricata* [224, 229]. Estas toxinas están presentes en las costas de Nueva Zelanda, Japón y Australia y recientemente se han encontrado en moluscos de Europa y Noruega [230]. Por otro lado, las PtTXs se aislaron en Japón de los bivalvos *Pteria penguin* [225] pero no han sido encontradas en Europa. El origen de las PnTXs y PtTXs es todavía desconocido pero se sospecha que son de origen marino, probablemente los dinoflagelados, en base a su alto grado de similitud estructural y características comunes con los SPXs y las DTXs [231, 232]. Sin embargo, los

prorocentrólidos y espiro-prorocentriminas son producidas por los dinoflagelados del género *Prorocentrum* [226, 228].

Los SPXs y las GYMs fueron descubiertas en la década de los 90, durante la vigilancia rutinaria de toxinas lipofílicas en los extractos de moluscos bivalvos en Canadá y Nueva Zelanda respectivamente [222, 223]. Desde entonces han tenido una distribución global. Así las GYMs se han detectado también en Túnez y Australia y los SPXs en Dinamarca, Noruega, Mar Adriático, Francia, Irlanda, Escocia y España, entre otros lugares [233-239]. Los organismos productores de SPXs son los dinoflagelados del género *Alexandrium* (*A. ostenfeldii* y en los últimos años se apunta a *A. peruvianum*) [240, 241], mientras que *Karenia selliformis* es el productor de las GYMs [242].

En cuanto a su estructura, las GYMs son compuestos pentacíclicos formados por un anillo de 16 carbonos unido a otro anillo con el grupo imino (Figura 9A). Los SPXs poseen el grupo imino unido a un ciclohexano y a un sistema tricíclico con anillos eter (Figura 9B).

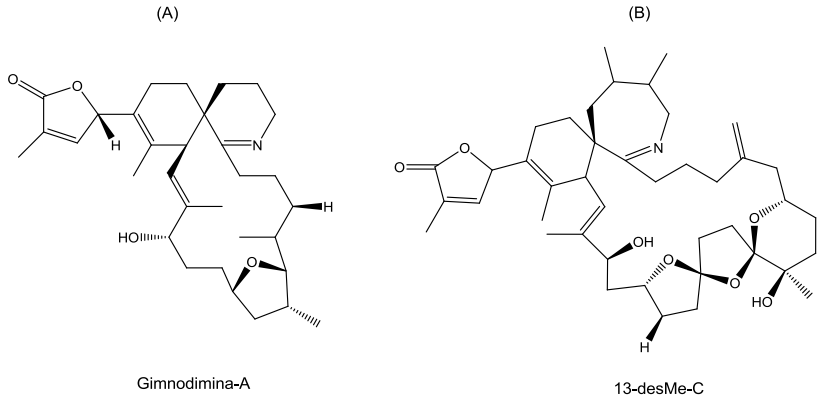


Figura 9: Principales estructuras de las gimnodiminas (A) y espirólidos (B).

Aunque su mecanismo de acción no se conoce completamente, se ha demostrado que la diana celular de las GYMs y SPXs son los receptores nicotínicos y muscarínicos de acetilcolina [243-245]. En la actualidad, estas toxinas no han sido relacionadas con intoxicaciones humanas, sin embargo debido a la alta toxicidad observada en el MBA por administración i.p., pueden suponer una amenaza real para la salud pública. Además pueden ser una fuente de falsos positivos en la detección de otras toxinas por MBA [246, 247].

Actualmente, estas toxinas se regulan estableciendo límites basados en la toxicidad oral en animales de laboratorio y la EFSA defiende el establecimiento de los ARfDs para los diferentes grupos de toxinas. Sin embargo, debido a la falta de suficientes datos cuantitativos sobre la toxicidad aguda por vía oral, no es posible [248]. Se han desarrollado varios métodos, alternativos al MBA para la detección de estas toxinas, como la técnica analítica LC-MS [193, 234, 249] y ensayos de fluorescencia de polarización [250-252].

1.2.2. Grupo de la Saxitoxina.

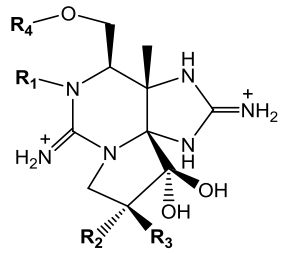
La saxitoxina (STX) y sus análogos constituyen un amplio grupo de alcaloides naturales neurotóxicos causantes del síndrome PSP, uno de los más peligrosos y extendidos del mundo.

Los eventos tóxicos de PSP han representado un impacto económico en áreas comerciales de moluscos de todo el mundo, desde Europa a Estados Unidos, Canadá y Chile [253]. En los últimos 40 años han tenido una frecuencia mayor y se han extendido a Sudáfrica, Marruecos, Australia, Nueva Zelanda, Japón, la India, Tailandia, Taiwán, Filipinas, China, Malasia, etc. [3, 254].

En ambientes marinos, las STXs son producidas principalmente por los dinoflagelados eucariotas del género *Alexandrium* (*A. tamarensis, A. minutum*, *A. catenella, A. andersoni, A. ostenfeldii, A. fraterculus, A. fundyense* y *A. cohorticula*), *Pyrodinium* (*P. bahamense var. Compressum*) y *Gymnodinium* (*G. catenatum*) [255, 256]. Aunque las STXs son detectadas mayoritariamente en moluscos bivalvos (ostras, mejillones, berberechos, vieiras y almejas), también se ha observado su presencia en crustáceos, gasterópodos [257], peces planctívoros (salmón, arenque, caballa) [258], algunos tipos de peces globo (*Tetraodon fangi*) y langostas [259, 260]. En agua dulce, las STXs son producidas por cianobacterias procariotas pertenecientes a los géneros *Anabaena* (*A. circinalis*) [261], *Aphanizomenon* (*A. flos-aquae*) [262], *Lyngbya* (*L. wollei*) [263, 264], *Cilindrospermopsis* y *Planktothrix* [265]. Las STXs de floraciones de cianobacterias provocan la contaminación de agua potable y aguas recreativas. Se han detectado altos niveles de toxinas en áreas de agua dulce de muchos países, como Australia, Brasil, Estados Unidos, México, Alemania y China [266-268]. Además se ha demostrado que algunas bacterias, aisladas de diferentes cepas tóxicas de especies de *Alexandrium* también

pueden producir toxinas del grupo de la STX [269, 270]. De hecho, en la Bahía de Ofunato, Japón, se atribuyó un incremento en la toxicidad de moluscos bivalvos en ausencia de dinoflagelados tóxicos a bacterias productoras de STXs [271]. Algunos autores sugieren por lo tanto, que las bacterias pueden actuar de forma autónoma y contribuir a la intoxicación de productos derivados de la pesca [272]. En este sentido se ha postulado que la producción de toxinas puede ser una función inherente de las bacterias marinas y necesaria en su proceso fisiológico [273].

Las STXs comprenden una gran familia de productos naturales marinos que contienen grupos guanidino, responsables de su alta polaridad [274, 275]. Se caracterizan por presentar estructuras similares pero cuya toxicidad difiere ampliamente. La más tóxica es la STX, su nombre proviene de la almeja amarilla, *Saxidomus giganteus* de donde se aisló por primera vez en 1957 [276]. En 1975 se sintetizó el primer derivado cristalino de la STX y se identificó su estructura [277, 278]. A partir de entonces más de 30 derivados se han identificado principalmente en dinoflagelados y moluscos que se alimentan de las microalgas tóxicas [8, 279]. En cuanto a la clasificación de los análogos de STX, hay varias categorías dependiendo de los autores, pero la más completa es la que reúne la existencia de 57 análogos descritos, colectivamente nombrados como toxinas paralizantes de molusco (PSTs) [265]. Los análogos más comunes son los hidrofílicos y los más ampliamente estudiados [280]. Todos ellos comparten un esqueleto en común de tetrahidropurina pero se diferencian por las combinaciones de hidroxilos y sulfatos en cuatro posiciones de la molécula R1-4 (Figura 10). Dependiendo de los sustituyentes en R4 las STXs se dividen principalmente en 3 grupos, que en orden descendente de toxicidad son: i) carbamato: STX, neosaxitoxina (neoSTX) y gonyautoxinas 1-4 (GTXs 1-4), ii) decarbamoil: decarbamoilsaxitoxina (dcSTX), decarbamoilneosaxitoxina (dcneoSTX) y decarbamoilgonyautoxinas 1-4 (dcGTXs 1-4) y iii) N-sulfocarbamoil: GTXs 5-6 y C 1-4 [8].



		R1	R2	R3	R4
Carbamato	STX	H	H	H	$CONH_2$
	GTX2	H	H	OSO_3^-	$CONH_2$
	GTX3	H	OSO_3^-	H	$CONH_2$
	neoSTX	OH	H	H	$CONH_2$
	GTX1	OH	H	OSO_3^-	$CONH_2$
	GTX4	OH	OSO_3^-	H	$CONH_2$
N-sulfo-carbamoil	GTX5	H	H	H	$CONHSO_3^-$
	C1	H	H	OSO_3^-	$CONHSO_3^-$
	C2	H	OSO_3^-	H	$CONHSO_3^-$
	GTX6	OH	H	H	$CONHSO_3^-$
	C3	OH	H	OSO_3^-	$CONHSO_3^-$
	C4	OH	OSO_3^-	H	$CONHSO_3^-$
Decarbamoil	dcSTX	H	H	H	H
	dcGTX2	H	H	OSO_3^-	H
	dcGTX3	H	OSO_3^-	H	H
	dcneoSTX	OH	H	H	H
	dcGTX1	OH	H	OSO_3^-	H
	dcGTX4	OH	OSO_3^-	H	H

Figura 10: Estructura de la saxitoxina y sus derivados.

Las STXs se unen de modo específico y reversible al sitio 1 de los canales de sodio dependientes de voltaje, situados en la cara externa del canal [281, 282]. Bloquean el flujo pasivo de iones sodio hacia el interior de la membrana impidiendo la conducción de los potenciales de acción y afectando la excitabilidad de los nervios y los músculos [283, 284]. Por esta razón el síndrome PSP se caracteriza por una serie de alteraciones neuromusculares que aparecen entre 5-30 minutos después de la ingestión de moluscos contaminados. Los síntomas dependiendo de la gravedad de la intoxicación, pueden ser leves como sensación de hormigueo o entumecimiento alrededor de los labios, extendiéndose a la cara y cuello, picazón en los dedos de la manos y pies, dolor de cabeza, mareos, náuseas y vómito. En intoxicaciones moderadas y severas se produce incoordinación motora, parestesia de brazos y piernas, dificultad respiratoria, incoherencia en el habla, parálisis muscular

creciente y finalmente insuficiencia respiratoria que puede llegar a causar la muerte [285].

Actualmente en la UE y en la mayoría de los países americanos y asiáticos, el límite máximo permitido para estas toxinas en moluscos destinados al consumo humano es de 800 µg STX eq/kg CM (cuerpo entero o partes comestibles) [109] y el método oficial de referencia para detectarlas es el MBA [286], aunque el método de HPLC-FLD de Lawrence [287, 288] puede ser utilizado oficialmente en la UE como una alternativa al método biológico. Los dos métodos han sido oficialmente validados en estudios interlaboratorio por la Asociación Oficial de Químicos Analíticos (AOAC) y ambos son capaces de detectar toxinas del grupo de la STX en el límite reglamentario.

El procedimiento del MBA fue desarrollado hace más de 50 años por Sommer y Meyer [289]. La AOAC lo modificó para contar con un método rápido y relativamente preciso para medir el contenido de toxinas PSP totales [290]. El método consiste en inyectar i.p. 1 mL del extracto ácido de los tejidos de moluscos a ratones de 20 g y monitorizar la sintomatología y el tiempo transcurrido hasta la muerte del animal. Si se trata de extractos muy tóxicos, estos se diluyen para que la muerte ocurra en un período de entre 5 y 15 minutos. La toxicidad de la muestra se expresa en MU (1 MU se define como la cantidad mínima necesaria para provocar la muerte de un ratón de entre 18 y 22 g de peso en 15 minutos) y se calcula según las curvas de respuesta a las dosis obtenidas con estándares de STX. El límite de detección del ensayo es aproximadamente 370 µg de STX eq/kg CM (la mitad del límite permitido), con una precisión ± 15-20%. En el MBA se observó que una alta concentración de sal en las muestras produce interferencias al disminuir la toxicidad aparente [291, 292], que la acumulación de zinc en las ostras provoca la muerte en ratones con síntomas neurológicos que se interpretan como causados por PSP [293] o que un pH demasiado ácido puede dar lugar a artefactos debidos a acidosis [253]. Además, existen limitaciones prácticas como un elevado coste (es necesario alimentar y mantener a ratones con un peso determinado), falta de sensibilidad (los límites de detección están a menudo cerca de los límites de regulación), falta de especificidad (las toxinas no se pueden identificar y cuantificar de forma individual) y una alta tasa de falsos positivos y negativos [253, 294]. A las dificultades encontradas se le suman consideraciones éticas y

legales sobre el uso de animales vivos en el laboratorio que han dado lugar a un incremento en la búsqueda de métodos alternativos.

En las últimas décadas se han desarrollado métodos inmunológicos, como ELISA y la inmunocromatografía de flujo lateral [295-297]. Ensayos de unión a receptores [298, 299], biosensores SPR [300, 301], sensores químicos basados en la transferencia de electrones fotoinducida [302] y biosensores de red neuronal [218]. Además de métodos electrofisiológicos [303-305] y ensayos fluorimétricos basados en cambios de potencial de membrana [306, 307]. Asimismo, las STXs también pueden ser detectadas mediante técnicas analíticas como la electroforesis capilar [308] y la LC [287, 309-314]. Mientras la mayoría de los métodos citados sólo informan de la toxicidad total de una muestra, la técnica de HPLC proporciona una información detallada del perfil de toxinas. Por ello, se considera el método de elección para la detección de las STXs. Debido a la naturaleza química de estas toxinas, que son altamente polares y carecen de un cromóforo sensitivo a la absorción de luz ultravioleta, la técnica de detección más frecuentemente utilizada en HPLC consiste en obtener un compuesto fluorescente, a través de una reacción de oxidación [310, 315, 316].

El método de HPLC-FLD con oxidación pre-columna, también llamado “método de Lawrence” y desarrollado durante los años 90, ha sido validado por la AOAC a través de un estudio interlaboratorial [287] y adoptado como método oficial en la legislación de muchos países [288] para la detección de toxinas PSP. Este método es aplicable para la determinación de STX, neoSTX, GTX2,3 (juntas), GTX1,4 (juntas), dcSTX, GTX5, C1 y C2 (juntas) y C3 y C4 (juntas) en moluscos (mejillones, almejas, ostras y vieiras). Las toxinas son extraídas de la carne de molusco homogenizada, por calentamiento con ácido acético y purificadas mediante extracción en fase sólida. Después de una reacción de oxidación con peróxido de hidrógeno y/o peryodato, los productos de los diferentes grupos de toxinas son separados por HPLC en fase reversa.

A pesar de que el método de Lawrence cumple los criterios de seguridad de equivalencia al MBA, el método presenta varios inconvenientes cuando se aplica en un entorno rutinario y legal [314]. De hecho, se han identificado problemas en el método como limitaciones en el rendimiento y la co-elución de productos de la oxidación [317]. Además, la falta de estándares de calidad

disponibles de todos los análogos hace que el método no esté validado para todas las toxinas del grupo de la STX. Estos inconvenientes, entre otros son un impedimento para su aplicación como método oficial de control de las STXs en la UE. Por lo tanto, este método sigue bajo observación en laboratorios de control de toda Europa y de hecho no se utiliza de forma rutinaria como único método para vigilar las zonas de producción de moluscos [318].

Por otro lado, el método de HPLC-FLD con oxidación post-columna propuesto por Oshima [310, 319] ha sido empleado durante años para la detección y cuantificación individual de cada una de las toxinas PSP. Este método, sometido a continuas modificaciones [312, 314] emplea 3 sistemas isocráticos en función del grupo de toxinas que se quieren separar. Por un lado emplea ácido heptanosulfónico en un tampón de fosfato de amonio, además de acetonitrilo para la separación de las toxinas carbamato (STX, dcSTX, neoSTX y dcneoSTX), ácido heptanosulfónico en un tampón de fosfato de amonio para la separación de las GTXs 1-6 y dcGTXs 1-4 y tetrabutilamonio en el mismo tampón para la separación de las toxinas Cs (C1-4). Las toxinas son separadas en una columna de fase reversa antes de la oxidación y la detección fluorimétrica. Además, se utiliza ácido peryódico en un tampón de fosfato de potasio para oxidar las toxinas y ácido acético para neutralizar la reacción de oxidación [223, 311, 320].

Los métodos de Lawrence y Oshima han demostrado su capacidad como posibles alternativas al MBA. Estos métodos miden de una manera efectiva el contenido de toxinas en los tejidos de los moluscos, que contienen una gran variedad de análogos. Por ello, muchos laboratorios los utilizan como métodos de cribado y para la reducción de animales en los MBA.

La base de datos toxicológicos de las STXs es limitada y comprende en su mayoría estudios sobre su toxicidad aguda tras la administración i.p. En vista de esto, y para fines de vigilancia utilizando el método de HPLC-FLD se han aplicado factores de toxicidad equivalentes para expresar los datos analíticos de los análogos detectados como equivalentes de STX [321]. Basándose en la toxicidad aguda, la EFSA estableció una ARfD de 0,5 µg STX eq/kg PC y para no exceder esta dosis, una porción de 400 g de CM no debería contener más de 75 µg STX eq/kg CM. Así mismo, la EFSA apuntó que no es posible hacer una estimación fiable del riesgo que supone el consumo de marisco disponible

en el mercado. Principalmente porque hay una alta proporción de muestras en las cuales no se detecta una contaminación con STXs. Esto es debido a los altos límites de detección de los métodos analíticos aplicados en los diferentes países de la UE. Además, las diferencias en la extracción de los moluscos por el MBA y el HPLC-FLD de Lawrence podrían dar lugar a la conversión de los análogos con baja toxicidad en análogos con alta toxicidad y por lo tanto originar diferentes resultados cuando los datos analíticos son expresados en STX equivalentes.

1.2.3. Grupo de la Tetrodotoxina

La tetrodotoxina (TTX) es una potente neurotoxina que se encuentra principalmente en las vísceras de muchas especies de peces tetraodóntidos y dióntidos, como el pez globo [322]. La mayoría de los casos de intoxicación alimentaria por TTX se observan en el suroeste asiático, principalmente en Japón [205], donde el “fugu” o pez globo representa una pieza importante en la cultura culinaria. Por este motivo el Ministerio japonés de Salud, Trabajo y Bienestar publicó una guía para peces globo comestibles en 1983, con actualizaciones en 1993 y 1995 [322, 323]. Desde entonces, los accidentes en restaurantes especializados han desaparecido, sin embargo muchos casos de envenenamiento por pez globo se producen cada año debido al consumo de platos preparados en casa con porciones tóxicas de los peces, como el hígado y los órganos reproductores. Estos materiales son preparados con pescado salvaje que se pesca de forma recreativa. Por otro lado, en Taiwán y China, donde está prohibido el consumo de pez globo, también se han producido intoxicaciones por TTX debidas al consumo, mayoritariamente de pequeños gasterópodos [324, 325]. Otros casos de intoxicaciones alimentarias han ocurrido en Tailandia, Bangladesh, Malasia, Hong Kong, Singapur, Australia, Madagascar, México y Estados Unidos [205, 326, 327].

Aunque la TTX existe sobre todo en aguas tropicales de todo el mundo, esta toxina también ha aparecido en las costas Europeas [328]. Esto se debe posiblemente a la migración lessepsiana, nombre por el que se conoce la entrada a través del canal de Suez de especies marinas procedentes del océano Indo-Pacífico hacia el mar Mediterráneo. Este fenómeno ha permitido a especies tóxicas asentarse en el Mediterráneo y plantear un problema de

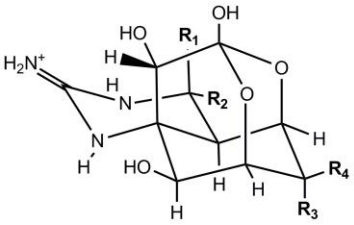
intoxicación alimentaria por el alto riesgo de mortalidad sobre todo si contienen TTX [329, 330]. Otra posibilidad podría ser un aumento en la temperatura del agua como consecuencia del calentamiento global. Como resultado, las especies marinas típicas de aguas tropicales y subtropicales, que contienen la TTX se adaptan a aguas menos cálidas y a cambios en las condiciones medioambientales y dan lugar a intoxicaciones en nuevas áreas geográficas. De hecho, se han descrito casos recientes de intoxicación por TTX en la costa Israelí [331, 332].

Durante muchos años se creía que la TTX se encontraba exclusivamente en el pez globo, bien de una manera endógena (producida por el propio pez) o exógena (tomada del exterior y acumulada en el pez). Desde que en 1965 se identificó la TTX en los huevos del tritón *Taricha torosa* [333], la TTX ha sido detectada en una amplia variedad de animales de diferente filo, incluyendo artrópodos, equinodermos, moluscos, gusanos (platelmintos y nemertinos), tritones y ranas [322, 334]. Además se ha identificado en dinoflagelados de la especie *Alexandrium tamarense* [335] y también en algunas cianobacterias de agua dulce [336].

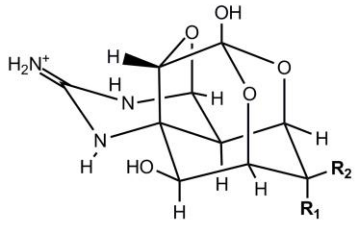
En el año 1980, varios estudios se centraron en la búsqueda del origen primario de la TTX en la cadena alimentaria, y se observó que esta toxina podría ser producida por ciertas especies de bacterias marinas: *Vibrio alginolyticus*, *Shewanella alga* y *Alteromonas tetraodonis*. Estas bacterias han sido aisladas de organismos que contenían TTX, tales como peces globo [337], estrellas de mar [338], el cangrejo xántido *Atergatis floridus* [339] y el alga roja *Jania* sp. [340, 341]. Por todo esto se consideró que la TTX es de origen bacteriano y se acumula en diferentes organismos a través de la cadena alimentaria. Estos organismos, muestran una alta resistencia a la toxina y parecen poseerla como un mecanismo de defensa biológico [342].

La TTX fue aislada por primera vez en 1950 como un prisma cristalino a partir de peces globo tóxicos [343] y su estructura fue elucidada por tres grupos diferentes en 1964 [344, 345]. Posee una estructura heterocíclica rígida que contiene diversos hidroxilos y un grupo guanidinio cargado positivamente a pH fisiológico. Actualmente se conocen más de 12 análogos de la TTX (Figura 11) [345, 346]. Algunos se encuentran químicamente en equilibrio con la TTX por la

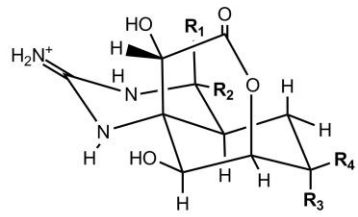
conversión que sufre esta toxina en el animal [347-349]. Otros, sin embargo se acumulan en los tejidos de forma natural [350-354].



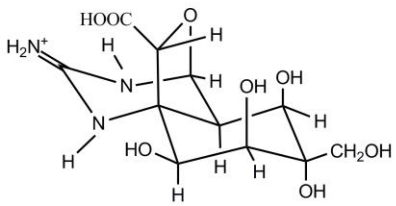
	R1	R2	R3	R4	MH^+ (*m/z*)
TTX	H	OH	OH	CH_2OH	320
4-*epi*TTX	OH	H	OH	CH_2OH	320
6-*epi*TTX	H	OH	CH_2OH	OH	320
11-deoxyTTX	H	OH	OH	CH_3	304
6,11-dideoxyTTX	H	OH	H	CH_3	288
11-norTTX-6(S)-ol	H	OH	OH	H	290
11-norTTX-6(R)-ol	H	OH	H	OH	290
11-norTTX-6,6-diol	H	OH	OH	OH	306
11-oxoTTX	H	OH	OH	$CH(OH)_2$	336



4,9-anhydroTTX	OH	CH_2OH			302
6-*epi*-4,9-anhydroTTX	CH_2OH	OH			302



5-deoxyTTX	H	OH	OH	CH_2OH	304
5,6,11-trideoxyTTX	H	OH	H	CH_3	272
4-*epi*-5,6,11-trideoxyTTX	OH	H	H	CH_3	272



tetrodonic acid	320

Figura 11: Estructura de la tetrodotoxina y varios de sus análogos.

Al igual que la STX, la TTX bloquea los canales de sodio de las células afectando a la transmisión nerviosa y a la contracción muscular [355-357]. Los síntomas de la intoxicación por TTX incluyen adormecimiento de los labios, la lengua, parestesia en la cara y extremidades, seguida de una sensación de mareo, dolor de cabeza y de estómago, náuseas, diarrea y vómito. En los casos más graves se puede producir inconsciencia, parálisis respiratoria, convulsiones y muerte [358].

La toxicidad tanto de los peces globo como de los gasterópodos, moluscos u otros animales marinos puede estar afectada por un cambio en las condiciones marinas, tales como elevaciones de la temperatura del agua debido al calentamiento global. Así mismo, especies marinas típicamente restringidas a los trópicos o subtrópicos se están encontrando en zonas norteñas. Debido a la migración de estas especies, los acuerdos internacionales de pesca de la UE podrían ser modificados. En principio la importación del pez globo y otras especies tóxicas no está permitida en algunos países, incluyendo Estados Unidos y Europa, y todavía no se han establecido límites reglamentarios para la TTX y sus análogos [205]. Con respecto a la legislación actual de la UE, los peces tóxicos pertenecientes a la familia tetraodontidae, o productos derivados, no deben ser colocados en los mercados europeos [50]. No obstante hay regulaciones para la TTX en países como Japón y Korea. Debido a que la toxina sólo se acumula en algunas partes del pescado, en estos países el control oficial de la TTX no regula la cantidad de toxina en el pescado que se puede colocar en el mercado, sino que exige licencias especiales a los restaurantes que quieran servir especies que contengan TTX.

La EFSA no ha publicado ningún documento relacionado con los riesgos que suponen las TTXs, pero algunos autores afirman que la dosis letal mínima en humanos es de 2 mg, aunque puede variar con factores como la edad, salud y sensibilidad a la toxina [323, 359]. Actualmente no existe un método oficial para la detección de estas toxinas, sin embargo el MBA se ha utilizado en muchos casos para determinar su toxicidad [360]. Otros métodos biológicos han sido desarrollados para la detección de TTX como inmunoensayos, ELISA [361, 362], ensayos con cultivos de neuroblastoma, ensayos hemolíticos [363] y además técnicas que utilizan biosensores [364, 365]. No obstante, para obtener una información específica de una muestra como el perfil tóxico o la cantidad

de una toxina individual, se han desarrollado métodos químicos basados en HPLC-FLD [366]. Aunque estos métodos garantizan límites de detección bajos, existen diferencias en las intensidades de fluorescencia de algunos análogos de la TTX en comparación con la propia TTX, lo que causa problemas en la cuantificación [367]. Por esta razón se han desarrollado métodos de LC-MS y LC-MS/MS [209, 367-370]. Estas técnicas analíticas, junto con métodos de extracción apropiados han podido determinar la presencia de TTX, no solo en los restos de peces y otros animales sino también en la sangre y orina de pacientes intoxicados [371, 372].

1.3. Monitorización de toxinas marinas en el mar

La contaminación con toxinas producidas por microalgas planctónicas y bentónicas es un problema global de salud pública y de protección de la calidad de los moluscos [285]. Los episodios tóxicos afectan a muchos de los sectores industriales como los productores de moluscos, la industria conservera, plantas de depuración e industrias asociadas que trabajan con marisco fresco y congelado, restaurantes y el turismo, entre otros. De hecho muchos gobiernos están concienciados con este problema e intentan buscar una solución a las pérdidas económicas debidas a la presencia de toxinas marinas [212]. Muchos países, especialmente aquellos con importantes industrias en la acuicultura y la pesca, tienen hoy en día programas de control de toxinas marinas de acuerdo con los requisitos de seguridad de alimentaria, de manera que puedan exportar a los mercados de la UE [50]. Estas regulaciones incluyen la vigilancia de las especies de algas tóxicas y la monitorización de toxinas en los moluscos para que estos cumplan con los límites prescritos. Además, algunos países complementan los datos de toxicidad de fitoplancton y moluscos con el seguimiento de diferentes parámetros medioambientales, que junto con el tiempo y las condiciones oceanográficas, constituyen una herramienta válida de predicción.

El objetivo prioritario de un programa de seguimiento es proteger la salud pública y establecer un sistema de control de las biotoxinas en los productos destinados al consumo humano. Además, proteger los cultivos de peces y el mercado de productos marisqueros, constituyendo así una red de alerta

temprana de los episodios tóxicos con capacidad de predecir la iniciación, duración y desaparición de estos eventos.

El uso de moluscos para la monitorización de toxinas sigue siendo la mejor técnica para determinar si un producto es apto para el consumo humano. Esto siempre que se complemente con técnicas de detección que ofrezcan selectividad y sensibilidad para todos los tipos de toxinas. Sin embargo, este tipo de seguimiento incluye algunas desventajas, como: dificultades en la recogida de las muestras, manipulación y transporte hasta el laboratorio, interferencias analíticas debido a los efectos matriz, entre otros.

La vigilancia del fitoplancton se utilizó por primera vez en 1994 en el control de biotoxinas marinas en Nueva Zelanda e inmediatamente se tradujo en un ahorro sustancial en los costes del muestreo y análisis para la industria marisquera [373]. A partir de entonces ha demostrado ser un método eficaz en general, fiable y rentable de alerta temprana del desarrollo de las floraciones de algas tóxicas y ha facilitado la identificación de los productores de toxina [138, 374]. A pesar de que el seguimiento del fitoplancton ha aumentado el conocimiento de los eventos tóxicos, éste solo ofrece un breve panorama de la composición del plancton en un determinado tiempo y lugar. Con frecuencia es difícil establecer una clara correlación entre la presencia de fitoplancton tóxico y la contaminación de moluscos. Además, la convivencia de especies de algas tóxicas y no tóxicas dentro del mismo género, puede conllevar a alertas innecesarias. Asimismo, la vigilancia del fitoplancton es un trabajo laborioso y requiere de especialistas taxonómicos bien entrenados para la identificación definitiva de algunas especies.

El uso de muestreadores pasivos como un sistema para la vigilancia de la distribución de toxinas marinas podría superar algunas de estas cuestiones o desventajas ya que ofrece una respuesta integrada en el espacio y en el tiempo. Varios autores se centraron en demostrar que el seguimiento de la toxina mediante la adsorción en fase sólida, denominado SPATT (en inglés, solid-phase adsorption toxin tracking) tiene potencial para proporcionar un sistema de alerta temprana integrado en el tiempo [375]. La idea de este sistema se basa en la observación de que cuando hay bajos niveles de algas tóxicas en la columna de agua, se pueden detectar pequeñas cantidades de biotoxinas disueltas [139]. Se ha demostrado que hay una relación entre la

detección de toxinas disueltas adsorbidas en una resina sintética, las densidades celulares de fitoplancton en la columna de agua y las concentraciones más altas de toxinas en los mariscos [375].

El uso de un sistema de muestreo pasivo tiene las ventajas de su simplicidad y bajo coste (almacenamiento, transporte…). Así como, de un muestreo integrado en el tiempo y el espacio, simulando la adsorción de los mariscos y siendo eficaz en sitios donde estos no existen, por ejemplo en sitios de centinela. Además, las matrices son relativamente limpias lo que facilita el proceso de extracción y análisis. Asimismo, proporciona una información única sobre la aparición de nuevas toxinas, su persistencia ambiental y las variaciones en la producción y duración de los episodios tóxicos [376, 377]. Este sistema, acoplado con un análisis apropiado (LC-MS, ELISA), ofrece un método sensible y capaz de proporcionar una alerta temprana de los eventos de floraciones tóxicas. Aunque todavía se requieren estudios adicionales para evaluar su eficacia con algunos grupos de toxinas, como las CTXs, PlTXs o BTXs, así como determinar sustratos adecuados para su adsorción. Actualmente se sigue profundizando en el diseño e implementación de métodos efectivos de monitorización de toxinas para minimizar su impacto ambiental.

2. Objetivo

Los fenómenos de floraciones de algas nocivas son un problema mundial de salud pública debido a su elevada letalidad, al gran número de intoxicaciones alimentarias que producen y a las pérdidas económicas en el sector marítimo y pesquero. El incremento en la frecuencia de estas floraciones y la aparición de nuevas toxinas marinas, ha hecho necesario aumentar los programas de monitorización y el desarrollo de métodos de cuantificación eficaces que garanticen bajos limites de detección y sobre todo aseguren la protección del consumidor. Es, por tanto, necesario mejorar los sistemas de alerta temprana y de detección. El objetivo de la presente tesis doctoral es estudiar la presencia de TTX y sus análogos en muestras de gasterópodos y peces recogidos en diferentes lugares de las costas europeas, y desarrollar un sistema de adsorción pasiva para monitorizar toxinas PSP y toxinas lipofílicas, disueltas en la columna de agua. Estas toxinas se cuantificarán utilizando diferentes métodos de detección química.

3. Publicaciones

3.1. Presentación

En esta sección se presentan los resultados obtenidos en la tesis doctoral, que incluyen 6 artículos publicados y un artículo sometido a una revista y pendiente de publicación. Dichos resultados van acompañados de la descripción de la metodología científica utilizada y de las conclusiones obtenidas en cada caso. Las publicaciones que se presentan están relacionadas con la detección de toxinas marinas, principalmente del grupo de la TTX, STX y toxinas lipofílicas en diferentes muestras (moluscos, peces, dinoflagelados…), y con la monitorización de algunas de estas toxinas en el agua del mar.

De este modo los resultados obtenidos se agrupan en tres secciones que se resumen a continuación:

3.2. Sección I: Estudio de la presencia de TTX en productos de la pesca procedentes de las Costas Europeas. Diseño de un método de detección de la tetrodotoxina y sus análogos.

La TTX es un compuesto natural altamente neurotóxico que actúa bloqueando los canales de sodio dependientes de voltaje [355-357]. Tal y como se ha descrito en la introducción de esta memoria, la TTX es producida por bacterias marinas y se distribuye sobre una amplia variedad de animales acuáticos a través de la cadena alimentaria [342]. La intoxicación por consumo de peces contaminados con TTX ocurre habitualmente en países asiáticos [322, 324, 325], sin embargo se han registrado casos en la costa Israelí [332]. Asimismo, esta toxina ha sido detectada recientemente en pescado capturado en las costas europeas [328].

En 2008, una persona ingresó en el hospital, en Málaga con síntomas de parálisis muscular después de haber ingerido parte de la carne de una caracola. Aunque ésta fue adquirida en el mercado mayorista de Málaga, en realidad fue pescada en la costa sur de Portugal. En el departamento de Farmacología se recibieron muestras de esa caracola y se procedió a su análisis. Tras un MBA se pensó que la muestra estaba contaminada con toxinas PSP. Sin embargo después de un análisis por HPLC se concluyó que estas toxinas no eran responsables de la intoxicación. A continuación, por los síntomas observados tanto en el paciente como en el MBA se sospechó que la

muestra podía contener TTX. Por lo que se procedió a estudiar la presencia de esta toxina en las muestras de caracola así como en los fluidos del paciente. Además se desarrolló un método de LC-MS más complejo para poder identificar y separar diferentes análogos de la TTX en muestras de pescado.

A esta sección corresponden las siguientes publicaciones:
I.1: First toxicity report of tetrodotoxin and 5,6,11-trideoxyTTX in the trumpet shell *Charonia lampas lampas* in Europe.
I.2: Seafood intoxication by tetrodotoxin: First case in Europe.
I.3: Liquid chromatography-mass spectrometry method to detect Tetrodotoxin and Its analogs in the puffer fish *Lagocephalus sceleratus* (Gmelin, 1789) from European waters.

I.1: Primer caso de intoxicación con tetrodotoxina y 5,6,11-trideoxyTTX por consumo de un molusco procedente de las costas europeas.

Resumen

La TTX es una de las toxinas más potentes aisladas, que se ha encontrado en una amplia variedad de animales. En este trabajo, se identificó la presencia de TTX y uno de sus análogos en una caracola marina de la especie *Charonia lampas lampas* y en los fluidos de un paciente intoxicado por el consumo de ésta. Las toxinas se analizaron mediante MS, microscopía confocal, LC-MS y MBA.

Inicialmente se detectó la presencia de la TTX y el análogo 5,6,11-trideoxyTTX en el LC-MS con el espectrómetro de masas operando en el modo EMS (en inglés: Enhanced Mass Spectrum). En una segunda medición, con el MS operando en modo EPI (en inglés: Enhanced Product Ion) se confirmó la existencia de ambas toxinas en las muestras, mediante el estudio de los iones fragmentados de cada molécula.

La TTX y 5,6,11-trideoxyTTX se detectaron en la glándula digestiva de la caracola y también en la orina y la sangre del paciente. La concentración de la 5,6,11-trideoxyTTX detectada en las muestras mediante LC-MS fue tres veces superior a la cantidad de TTX. Sin embargo, los resultados obtenidos por MBA mostraron que el análogo es mucho menos tóxico que la TTX. En los

experimentos de toxicidad *in vitro* usando células de cerebelo, la muestra de caracola mostró una alta toxicidad, pero los niveles de toxina fueron menores que en los resultados obtenidos *in vivo*, probablemente debido a una cierta competencia entre ambas toxinas. Este trabajo muestra por primera vez la presencia y la toxicidad de TTX y 5,6,11-trideoxyTTX en una caracola recogida en las costas Europeas. El método de LC-MS utilizado, se considera una herramienta útil para confirmar la presencia de TTX y además para identificar la presencia de varios de sus análogos.

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First Toxicity Report of Tetrodotoxin and 5,6,11-TrideoxyTTX in the Trumpet Shell *Charonia lampas lampas* in Europe

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Tetrodotoxin (TTX) is one of the most potent toxins already isolated, which occurs in a wide variety of animals. In this work, the occurrence of TTX and analogues was examined using mass spectrometry, confocal microscopy, liquid chromatography−mass spectrometry (LC−MS), and mouse bioassay in a trumpet shell (*Charonia lampas lampas*) and in the fluids of a patient poisoned by consuming this shell. Retention time data in the LC−MS system within the enhanced mass spectrum (EMS) mode indicated the presence of TTX and the analogue 5,6,11-trideoxyTTX; the enhanced product ion (EPI) mode confirmed the existence of both toxins with the formation of characteristic daughter ions from the fragment pattern of each molecule. TTX and 5,6,11-trideoxyTTX were only detected in the digestive gland of the trumpet shell and also in the urine and serum of the patient. The concentration of 5,6,11-trideoxyTTX checked in the samples by LC−MS was 3 times higher than TTX. However, the results obtained by mouse bioassay showed that the analogue is much less toxic than TTX. In vitro toxicity was checked using cerebellar cells; in these experiments the trumpet shell sample showed high toxicity, but the level was lower than in vivo results probably due to some competition between analogues. This paper shows for first time the presence and toxicity of TTX and 5,6,11-trideoxyTTX in a trumpet shell collected in the European coasts. The LC−MS method is a useful tool to confirm the presence of TTX and the further identification of TTX analogues.

Tetrodotoxin (TTX) is one of the most potent neurotoxins and is known to block sodium ion channels responsible for nerve and muscle excitability.[1] Its molecule has six hydroxyl residues at the C-4, C-6, C-8, C-9, C-10, and C-11 positions in addition to a guanidinium group (Figure 1A), which is positively charged in the biological pH range. Although the hydroxyls at C-9 and C-10 are the most important, those at C-4, C-6, and C-11 also make significant contributions to the binding to the channel as hydrogen bond donors.[2]

TTX usually exists as a mixture of its analogues (TTXs) in puffer and other tetraodontiforme fish[3] and causes paralytic poisoning and occasionally death in humans through ingestion.[1,4] The symptoms included numbness of the tongue, lips, paresthesia of the face and limbs, followed by a sense of light-headedness and floating, head- and stomachache, nausea, diarrhea, and vomiting. In severe cases, there is unconsciousness, respiratory paralysis, and convulsions.[5]

Many cases of TTX-food poisoning are reported in Southeastern Asia and, more specifically, Japan.[6] In addition to Japan, Taiwan, and Thailand, intoxication has also been reported from the South Pacific, Malaysia, Hong Kong, Singapore, Australia, Madagascar, China, and Bangladesh.[6] Since the description of TTX, several studies revealed its wide distribution in terrestrial as well as marine animals including the Anuran family *Brachycephalidae*,[7] newts *Notophathalmus viridescens*,[8] *Triturus* spp.,[9] goby *Gobius criniger*, xanthid crab *Atergatis floridus*, blue-ringed octopus *Octopus maculosus*, chaetognatha, starfish *Astropecten polycanthus* and *A. scoparius*, four species of gastropods *Zeuxis siquijorensis*, *Babylonia japonica*, *Tutufa lissostoma*, and *Niotha clathrata*, three species of nemerteans *Tubulanus punctatus*, *Lineus fuscoviridis*, and *Cephalothrix linearis*, and the trumpet shellfish *Charonia sauliae*.[10]

Unlike the rest of biotoxins that accumulate in fishery products, the TTX is not produced by microalgae. Symbiotic bacteria have been suggested to be involved in TTX genesis for marine animals[9]

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(1) Kawatsu, K.; Shibata, T.; Hamano, Y. *Toxicon* **1999**, *37*, 325–333.

(2) Yotsu-Yamashita, M.; Sugimoto, A.; Takai, A.; Yasumoto, T. *J. Pharmacol. Exp. Ther.* **1999**, *289*, 1688–1696.

(3) Jang, J.; Yotsu-Yamashita, M. *Toxicon* **2006**, *48*, 980–987.

(4) Tsai, Y. H.; Hwang, D. F.; Cheng, C. A.; Hwang, C. C.; Deng, J. F. *J. Chromatogr., B* **2006**, *832*, 75–80.

(5) Ochoa, J. L.; Núñez-Vázquez, E. J.; Ortega, A. G.; Parra, I. A. d. l. *Panorama Acuícola Magazine* **2006**.

(6) Gessner, B. D.; McLaughlin, J. B. *Seafood and Freshwater Toxins. Pharmacology, Physiology, and Detection*, 2nd ed.; CRC Press: Boca Raton, FL, 2008.

(7) Pires, O. R., Jr.; Sebben, A.; Schawartz, E. F.; Morales, R. A. V.; Bloch, C., Jr.; Schwartz, C. A. *Toxicon* **2005**, *45*, 73–79.

(8) Yotsu-Yamashita, M.; Mebs, D. *Toxicon* **2001**, *39*, 1261–1263.

(9) Yotsu-Yamashita, M.; Mebs, D.; Kwet, A.; Schneider, M. *Toxicon* **2007**, *50*, 306–309.

(10) Wu, Z.; Xie, L.; Xia, G.; Zhang, J.; Nie, Y.; Hu, J.; Wang, S.; Zhang, R. *Toxicon* **2005**, *45*, 851–859.

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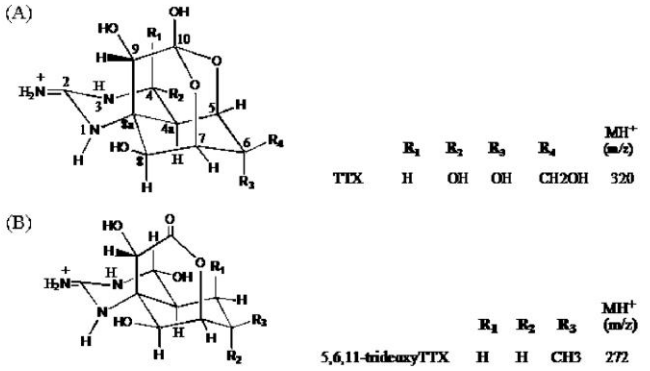

Figure 1. Chemical structures of TTX (A) and 5,6,11-trideoxyTTX (B).

and specifically have involved *Shewanella algae*, *S. putrefaciens*, *Vibrio* sp., *Pseudomonas* sp., and *Alteromonas tetraodonis* that accumulated in the subcutaneous mucus, or in the intestine, releasing the TTX,[11,12] which were later confirmed by the isolation of TTX-producing bacteria from different TTX-bearing animals.[10]

A food poisoning incident resulting from the ingestion of a trumpet shell of the species *Charonia lampas lampas*, involved a single person (49 year old man) in October 2007 (paper submitted). The symptoms began minutes after ingestion of the mollusk; symptoms included abdominal pain with nausea and vomiting, weakness, difficulty articulating words and keeping the eyelids open, and difficulty breathing. After 72 h, the symptoms were fully reversed. These symptoms were similar to those of TTX poisoning.

The species *Charonia lampas* (family: *Ranellidae*) is typical of the Western Mediterranean and the northeast Atlantic being very frequent in the Sea of Alborán (westernmost portion of the Mediterranean Sea, between Spain and Morocco). As a result of climate change and rising water temperatures, exotic species from the Red Sea or tropical habitats are colonizing the Mediterranean, and it is possible that TTX-bearing animals infected by TTX-producing bacteria have contaminated the species *Charonia lampas lampas* through the food chain.[13]

The identification and quantification of TTX and TTXs are important not only for food hygiene but also for the study of the biosynthetic pathway of TTX which has not yet been clarified.[14] In the present study, we report analytical and biological data on the presence of the TTX and 5,6,11-trideoxyTTX in the trumpet shell *Charonia lampas lampas* implicated in the human poisoning and the presence of these toxins in the urine and blood following the intoxication.

MATERIALS AND METHODS

Materials. TTX used as a calibrant was from Calbiochem Corporation. The HPLC grade methanol, acetonitrile, and acetic acid were from Panreac (Spain). Trimethylamine (25%) solution in water and ammonium formate were from Sigma (Spain). Formic acid was obtained from Merck (Spain).

The trumpet shell (24 cm shell length) was from the species *Charonia lampas lampas*, purchased in a Malaga market. Its harvest place was later traced back to the south coast of Portugal. The samples corresponding to the trumpet shell were sent from the Public Health Laboratory of Malaga.

Urine, blood, and serum samples were collected from the patient and frozen at −20 °C until the analysis was carried out. These samples were obtained to identify and describe the clinical case by Mr. Juan Francisco Fernández Ortega (paper submitted).

Samples of *Charonia lampas* and *Murex trunculus* were later collected at the same time from the Algarve offshore coast and Riá Formosa, respectively, at the south coast of Portugal. The samples were provided by the National Reference Laboratory of Marine Biotoxins (INRB/IPIMAR).

Sample Preparation of Trumpet Shell. The trumpet shell *Charonia lampas lampas* was boiled and then was divided into two parts: digestive gland (sample 1) and remaining tissues (sample 2). Both samples were extracted according to the official procedure for PSP (paralytic shellfish poisoning). Sample 1 was formed by a residue with pasty consistency and dark color that was the visceral mass at the end of the trumpet shell. In this sample (3 g) an initial 1/10 dilution to pH 2, adjusting the final volume to 30 mL with hydrochloric acid 0.1 M. Sample 2 was constituted of the meat of the trumpet shell after being crushed and homogenized. After homogenization, the sample (50 g) was extracted with 200 mL of hydrochloric acid to pH 3. An aliquot of 200 μL of each sample was vaccuum-dried and dissolved in 1000 μL of methanol. Aliquots were filtered through an Ultrafree-MC centrifugal filter (Millipore Corporation) and then analyzed.

The other samples of *Charonia lampas* and *Murex trunculus* were boiled for 15 min and then were dissected in visceral mass and remaining tissues. The samples (3−5 g) were extracted with 1% acetic acid and analyzed by LC−MS.

Biological Samples Preparation. The sample cleanup procedure used was developed before for TTX and saxitoxin analysis of biological samples.[4] Each sample of urine, blood, and serum (1.0 mL) was mixed with 500 μL of 0.5 M acetic acid. The samples were immediately centrifuged at 10 000*g* for 10 min. The super-

(11) Noguchi, T.; Arakawa, O.; Takatani, T. *Comp. Biochem. Physiol.* **2006**, 145–152.
(12) Croci, L.; Cozzi, L.; Suffredini, E.; Ciccaglioni, G.; Toti, L.; Milandri, A. *Harmful Algae* **2006**, *5*, 266–274.
(13) Gallitelli, M.; Ungaro, N.; Addante, L. M.; Procacci, V.; Silveri, N. G.; Sabba, C. *Jama* **2005**, *293*, 2599–2600.
(14) Shoji, Y.; Yotsu-Yamashita, M.; Miyazawa, T.; Yasumoto, T. *Anal. Biochem.* **2001**, *290*, 10–17.

natant was passed through a cartridge column (Supelclean LC-18 cartridges; Supelco) preconditioned with 10 mL of methanol followed by 10 mL of water. After the sample was applied into the cartridge, it was eluted with 10 mL of acetic acid (0.3%). The eluant was freeze-dried, dissolved in 2 mL of acetic acid (0.3%), and filtered through a 10 000 NMWL cutoff Ultrafree-CL centrifugal filter (Millipore Corporation). The filtrate was freeze-dried and dissolved in 250 μL of methanol and analyzed by LC−MS.

Confocal Microscopy Analysis. *Cell Cultures.* Primary cultures of CGC were obtained from cerebella of 7-day-old mice following previously described methods.[15,16] In brief, cells were dissociated by mild trypsinization with trypsin from bovine pancreas (0.002% w/v) at 37 °C, followed by trituration in a DNase solution (0.004% w/v) containing a soybean trypsin inhibitor (0.05% w/v). The cells were suspended in DMEM containing 25 mM KCl, 31 mM glucose, and 0.2 mM glutamine supplemented with *p*-amino benzoate (0.1% w/v), insulin (0.04% w/v), penicillin (0.03% w/v), and 10% fetal calf serum. The cell suspension was seeded in glass coverslips precoated with poly-L-lysine and incubated in 6-multiwell plates for 7−11 days in a humidified 5% CO_2/95% air atmosphere at 37 °C. Cytosine arabinoside, 20 μM, was added before 48 h in culture to prevent glial proliferation.

Determination of Plasma Membrane Potential (E_m). The coverslips plated with CGC were transferred to custom-made recording chambers, in which the cells were loaded with 20 nM [$DIBAC_4(3)$], to monitor the E_m.[17,18] In order to calculate the ability of different TTX concentrations and the TTX-containing samples to block voltage-activated sodium channels, the intensity of the fluorescence was evaluated after exposure of the neurons to veratridine alone (control values) or to veratridine after preincubation with different concentrations of TTX or the samples of trumpet shell (samples 1 and 2) evaluated in this work.

The $DIBAC_4(3)$ fluorescence was monitored using a Nikon C1 confocal microscope and Nikon Plan Apo Tirf 60×, NA 1.45 objective (Nikon, Melville, NY). The images of fluorescence emitted at 515 nm (after excitation at 488 nm) were collected every 5 s. The fluorescence intensities, measured in selected regions of interest, were analyzed off-line using the Nikon EZ-C1 viewer software.

High-Performance Liquid Chromatography−Mass Spectrometry (LC−MS) Analysis. The high-performance liquid chromatography (LC) equipment was formed by a binary system of LC-10ADVP pumps, an autoinjector (SIL-10ADvp) with refrigerated rack, degasser, column oven, and the system controller from Shimadzu (Japan). This system was coupled to a mass spectrometer (MS) QTRAP-2000 instrument from Applied Biosystems, which consists of a hybrid quadrupole-linear ion trap mass spectrometer equipped with an electrospray (ESI) source. The nitrogen generator is a Nitrocraft $NC_{LC/MS}$ from Air Liquide (Spain). The separation and identification of toxin was achieved in a Zorbax 300SB-C3 column (i.d. 4.6 mm × 150 mm) inside the column oven at 25 °C. The injection volume was 5 μL. The mobile phase for analysis was 1% acetonitrile, 10 mM trimethylamine (TMA), and 10 mM ammonia formate (pH 4.0 with formic acid) at an isocratic flow rate 0.4 mL/min. Analyst software was used for instrument control as well as data processing and analysis.

Extracts were analyzed with the ESI interface operating in the positive ion mode using the following parameters: curtain gas, 25; CAD gas, 6; IonSpray voltage, 4000 V; temperature, 500 °C; gas 1, 50; gas 2, 50; these parameters had been previously optimized using the toxin standard. The mass spectrometer was operated in the enhanced mass spectrum (EMS) mode to confirm the presence of TTX and in the enhanced product ion (EPI) mode to quantify the toxin. For EPI positive, the transitions selected were TTX, 320 → 302/320 → 162 (m/z range 2 amu).

Mouse Toxicity Assay. The lethal potency of samples of the trumpet shell was estimated by intraperitoneal (i.p.) injection of the extract or TTX standard into mice (19−23 g of body weight). The toxicity was determined by the time of death of six mice, according to the standard dose−lethal time plot prepared by using the commercial TTX. The amount of toxin was expressed in mouse units (MU, mean ± SD); one MU is defined as the amount of toxin required to kill an 18−23 g ICR mouse in 7−15 min after i.p. administration.[5,10]

RESULTS

The trumpet shell *Charonia lampas lampas* involved in the poisoning episode was purchased in the Malaga market, and its collection later traced back to the south coast of Portugal. It was sent to the Public Health Laboratory of Malaga, and there the trumpet shell was dissected into the digestive gland (sample 1) and the remaining tissues (sample 2). These samples were initially analyzed by mouse bioassay according to PSP protocol, as TTX was never reported in Europe before. The PSP concentration value obtained in sample 2 was 151 μg of saxitoxin equiv/100 g of flesh. Sample 1 checked by bioassay showed a very high PSP concentration (25 500 μg of saxitoxin equiv /100 g). To be able to quantify the samples, the extract was diluted 1/100 with buffered saline solution to pH 3.0. The time of death of the mice was approximately 7 min 10 s and 7 min 15 s, which indicated a very high toxicity.

Subsequently both samples were analyzed by the Lawrence[19] and Oshima[20] methods in the Community Reference Laboratory of Marine Biotoxins-CRLMB of Vigo and in the Department of Pharmacology of Lugo, respectively. The analyses indicated a total absence of PSP toxins by both methods. Because of the toxicity observed in the mouse bioassay and the patient symptoms, it was suspected that the samples could have TTX.

Samples of *Charonia lampas* and *Murex trunculus* collected from the south coast of Portugal were also analyzed by the Lawrence method[19] at IPIMAR laboratory, and PSP toxins were not detected although some traces appeared at the limit of detection.

Then, all samples were analyzed by mass spectrometry to check for the presence of TTX. The TTX standard solutions and samples of trumpet shell were dissolved in methanol and then

(15) Schousboe, A.; Meier, E.; Drejer, J.; Hertz, L. In *A Dissection and Tissue Culture Manual of the Nervous System*; Shahar, A., de Vellis, J., Vernadakis, A., Haber, B., Eds.; Alan R Liss: New York, 1989; pp 203−206.
(16) Vale, C.; Nicolaou, K. C.; Frederick, M. O.; Gomez-Limia, B.; Alfonso, A.; Vieytes, M. R.; Botana, L. M. *J. Med. Chem.* **2007**, *50*, 356–363.
(17) Vale, C.; Alfonso, A.; Sunol, C.; Vieytes, M. R.; Botana, L. M. *J. Neurosci. Res.* **2006**, *83*, 1393–1406.
(18) Vale-Gonzalez, C.; Pazos, M. J.; Alfonso, A.; Vieytes, M. R.; Botana, L. M. *Toxicon* **2007**, *50*, 541–552.
(19) Lawrence, J. F.; Ménard, C.; Cleroux, C. *J. AOAC Int.* **1995**, *78* (2), 514–520.
(20) Oshima, Y. *J. AOAC Int.* **1995**, *78*, 528–532.

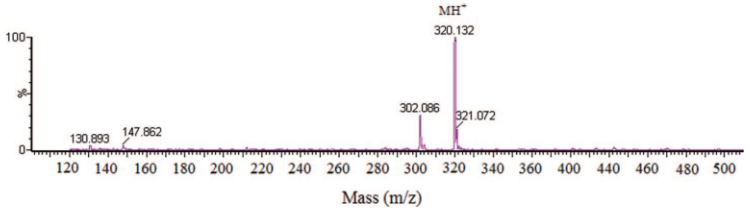

Figure 2. Mass spectrometry analysis by infusion of TTX standard.

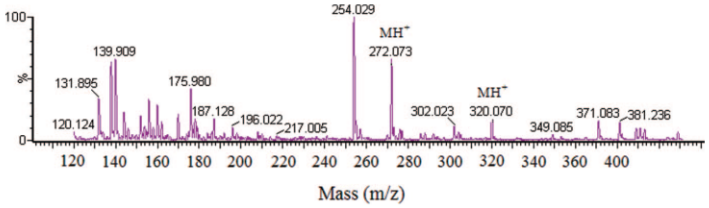

Figure 3. Mass spectrometry analysis by infusion of sample 1; the protonated molecular ion MH^+ of TTX was *m/z* 320 and of 5,6,11-trideoxyTTX was *m/z* 272.

directly injected into the MS system by infusion. A TTX standard was first injected, and a peak at *m/z* 320 was observed (Figure 2). The injection of a sample 1 extract provided two peaks, one at *m/z* 320 that corresponds to TTX and another peak at *m/z* 272 that corresponds to the analogue 5,6,11-trideoxyTTX[14] (Figure 3). However when sample 2 was injected, TTX and 5,6,11-trideoxyTTX were not detected.

Once we have confirmed the presence of TTX in the *Charonia* sample, the amount of toxin was quantified by LC−MS. The molecular ions corresponding to the $(M + H)^+$ ions of TTX (*m/z* 320) and 5,6,11-trideoxyTTX (*m/z* 272) were detected in the EMS and EPI mode by LC−MS. With the TTX standard, a calibration curve with six concentrations of TTX (10−1000 ng/mL) versus the EPI signal was done to transform the signal obtained in each sample to TTX concentration. Figure 4A shows the LC−MS chromatogram of a TTX standard, with only one peak at the retention time of 4.5 min. The mass spectrum of this peak is shown in Figure 4B where a high peak with *m/z* 320.1 is observed corresponding to the mass of TTX. Then this injection was repeated in the EPI mode; as shown in parts C and D of Figure 4 (LC−MS/MS chromatogram) with the formation of characteristic daughter ions 302.1, 284.3, 256.1, 178.0, and 162.1. In the same conditions, the samples of trumpet shell and the samples of urine and blood were analyzed. The LC−MS/MS chromatogram of sample 1, Figure 5A, shows a peak at retention time 4.5 min that corresponds to TTX; the mass spectrum of this peak, Figure 5B, shows the parent peak at *m/z* 320 and the daughter peaks *m/z* 302 and 162 indicating the presence of TTX. Figure 5C shows a peak at retention time 5.6 min that corresponds to 5,6,11-trideoxyTTX; the mass spectrum of this peak, Figure 5D, shows the parent peak at *m/z* 272 and the daughter peaks *m/z* 254 and 162 confirmed the presence of 5,6,11-trideoxyTTX. The same spectra were obtained after urine injection, Figures 6A−D. In addition, when the blood serum was injected, the same peaks were detected even though the signal was close to the detection limit. On the contrary, in sample 2, both toxins were again not detected. Samples of *Charonia lampas* and *Murex trunculus* collected by IPIMAR were also analyzed at the same conditions, but the analysis indicated total absence of TTX and TTXs.

The results obtained by LC−MS were transformed into the amount of TTX by using a calibration curve done with the transitions of the daughters ions (*m/z* 302 and 162) obtained in the EPI mode. Therefore, the amount of TTX in the samples calculated with these transitions was 31.5 mg/100 g in sample 1, 211.1 ng/mL in the urine sample, and 26.4 ng/mL in the serum sample. The quantification of 5,6,11-trideoxyTTX was calculated using the TTX pattern as a standard due to the unavailability of standards for this compound and assuming a relative response factor of 1:1. Since the analogue has different transitions than that of TTX, its quantification was done in the EMS mode with reference to the TTX peak. At these conditions, the amount of 5,6,11-trideoxyTTX was 100.4 mg/100 g in sample 1, 692.1 ng/mL in the urine sample, and 86.5 ng/mL in the serum sample. These results are shown in the Table 1.

In order to quantify the toxicity of the samples by a nonanimal assay, samples 1 and 2 from *Charonia lampas lampas* were analyzed by an in vitro functional assay. The purpose was to quantify the presence of TTX by a rapid and sensitive in vitro model previously employed to detect the presence of paralytic shellfish toxins[21] by measuring the ability of these types of toxins to block the veratridine-induced changes in membrane potential in primary cultured neurons. Veratridine is known to depolarize excitable cells by opening the voltage-dependent sodium channels and blocking its inactivation whereas TTX toxins block neuronal transmission through inhibition of voltage-gated Na^+ channels. Hence, the addition of veratridine (50 μM) to excitable cells increases $DIBAC_4(3)$ fluorescence and preincubation of the cells with TTX before addition of veratridine reduces the veratridine-induced depolarization. Figure 7 shows that TTX standard blocked the veratridine-induced depolarization with an IC50 of 4.7×10^{-9} M (95% confidence intervals from 3.2 to 6.8×10^{-9}M). In this in vitro model, sample 1 inhibited the VTD-induced depolarization

(21) Vale, C.; Alfonso, A.; Vieytes, M. R.; Romaris, X. M.; Arevalo, F.; Botana, A. M.; Botana, L. M. *Anal. Chem.* **2008**.

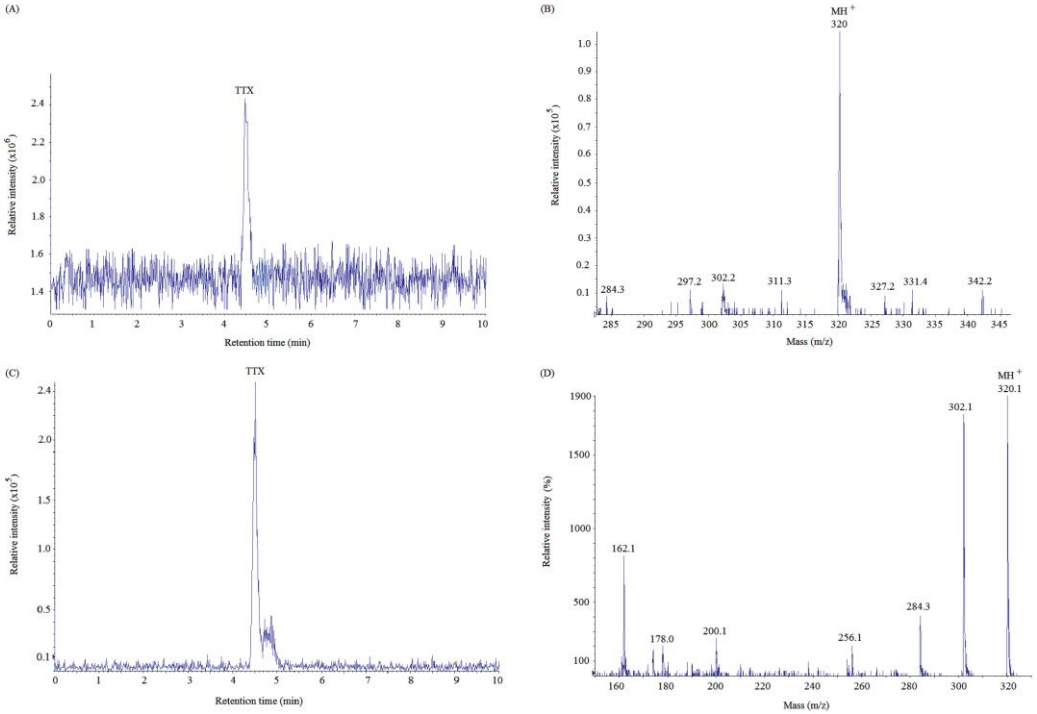

Figure 4. LC−MS analysis of a TTX standard. (A) LC−MS chromatogram of TTX obtained in the EMS mode. (B) LC−MS mass spectrum of TTX from part A. (C) LC−MS/MS chromatogram of TTX obtained in the EPI mode. (D) LC−MS/MS mass spectrum of TTX from part C.

by 33.3 ± 7.2%, which will be equivalent to the presence of approximately 10 nM TTX in the diluted sample, therefore equivalent to 3.2 mg in 100 g of tissue; whereas sample 2 did not show any effect on the veratridine-induced depolarization.

Finally, the toxicity of the samples was also determined by mouse bioassay. The equivalence was determined in μg/mL of TTX, in MU. Six mice injected i.p. with the extract of trumpet shell (1 mL/mouse) showed typical symptoms similar to those described for TTX and their analogue toxins in mice (paralysis of the hind legs and brief dyspnea), and in approximately 7 min they died. The TTX standard was injected, and the concentration of TTX in MU was equal to 0.355 ± 0.03 μg/mL of TTX. Then sample 1 was diluted 1:100, and a volume of 1 mL was injected into mice; the toxicity determined by the mouse bioassay was 1.53 MU. The concentration of toxin by LC−MS in this volume was 0.380 μg/mL of TTX and 1.213 μg/mL of 5,6,11-trideoxyTTX; 0.380 μg/mL of TTX produces 1.07 MU, therefore, the toxic value attributed to the analogue is 0.460 MU. These results are shown in Figure 8. The mouse bioassay evidences that the analogue is less toxic than TTX, and therefore it would require a concentration 3 times higher than that of 5,6,11-trideoxyTTX to show half of the toxicity of TTX.

DISCUSSION

LC−MS is a known and trusted technology to detect and quantify toxins in any biological sample.[6] With the use of this technique, high amounts of TTX and 5,6,11-trideoxyTTX were detected in both the digestive gland of the trumpet shell and in the patient body fluids. The percentage of toxin in urine versus blood after 33 h (paper submitted) was 90%. Similar results and technology were reported in other TTX human intoxication after fish consumption.[4] The rapid recuperation of the patient is more reliable with the elimination of TTX in urine, indicating that TTX is redistributed from the serum to its target sites at sodium channels.[22] Urine is the major excretion route of TTX intoxication and of PSP intoxication for human beings.[23]

From the results in the present study, it is suggested that the combination of a protocol that includes precleanup of the samples with LC−MS analysis is very useful in detecting TTX from urine samples of poisoned patients for diagnosis of TTX-food poisoning, as it had been previously reported for saxitoxin.[4]

The analysis done by confocal microscopy on plasma membrane potential changes indicated an amount of 3.2 mg of TTX/100 g of tissue. The fact that sample 1 shows a lower toxicity by this analysis than those obtained by LC−MS and mouse bioassay could be because the cell assay is underestimating the amount of toxin found in the sample, due to limitations of the fluorescent probe. However, another possibility is that 5,6,11-trideoxyTTX may

(22) O'Leary, M. A.; Schneider, J. J.; Isbister, G. K. *Toxicon* **2004**, *44*, 549–553.
(23) Gessner, B. D.; Bell, P.; Doucette, G. J.; Moczydlowski, E.; Poli, M. A.; Van Dolah, F.; Hall, S. *Toxicon* **1997**, *35*, 711–722.

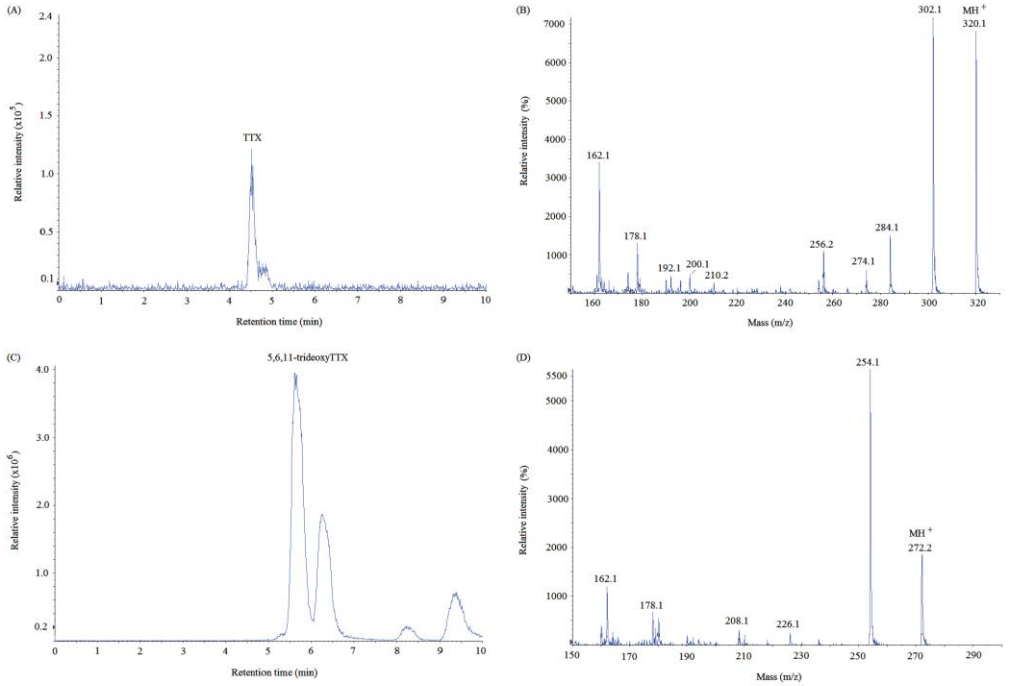

Figure 5. LC−MS/MS analysis in the EPI mode of sample 1. (A) LC−MS/MS chromatogram of sample for *m/z* 320. (B) LC−MS/MS mass spectrum from part A. (C) LC−MS/MS chromatogram of sample for *m/z* 272. (D) LC−MS/MS mass spectrum from part C.

be a partial agonist to TTX on the binding to the sodium channel, which induces a loss of TTX effect on the intracellular target. Also in the in vitro analysis we are isolating cerebellar cells, while in the mouse bioassay the whole body can be a target for TTX, so it is difficult to get similar results of toxicity by both methods. It is important to highlight the fact this paper reports for the first time this membrane potential assay as a nonanimal alternative method to detect TTX.

In the analysis carried out by LC−MS; the fragment ions at *m/z* 302 and 284 of TTX obtained by LC−MS/MS were due to elimination of one or two water molecules, respectively, but the ion at *m/z* 256 probably generated with the loss of 28 mass units from the ions of $[MH - H_2O]^+$ or $[MH - 2H_2O]^+$, could be accounted for by elimination of CO at C-10 as the result of the cleavage of the bonds between C-9 and C-10, C-10 and C-5−O, and C-10 and C-7−O, due to the α-hydroxy hemilactal structure.[14] The fragment ion at *m/z* 254 of 5,6,11-trideoxyTTX is due to the loss of one water molecule. In contrast, the specific fragment ions at *m/z* 162 and 178 that appeared on the spectra of TTX and 5,6,11-trideoxyTTX can be interpreted as 2-aminohydroxiquinazolines and 2-aminodihydroxyquinazolines, respectively (Figure 9). These structures probably are due to bond cleavage between C-8a and C-9, and between C-6 and C-11.[7,14]

TTX and 5,6,11-trideoxyTTX were detected in the digestive gland of the trumpet shell *Charonia lampas lamas*; however, both toxins were not detected in the others tissues of the trumpet shell. This agrees with previous results in other study where TTX was only detected in the digestive gland of the species *Charonia sauliae*.[11]

In the *Charonia lampas* sample collected by IPIMAR at the south coast of Portugal and unrelated to the poisoning episode, TTX and TTXs were not detected. As in the sales documents, the whelk species was referred to as *Murex* spp., samples of *Murex trunculus* (now *Hexaplex trunculus*) were also included in the analysis, but no TTXs were detected. The question remains about the exact origin of TTX in the food chain, because the ecological environments of TTX-bearing animals seem to have no common factor other than being closely implicated in an aquatic system; bacteria, the omnipresent organisms that commonly inhabit aquatic systems, were implicated as the primary source of TTX.[11] Also, if contamination exists in Mediterranean *C. lampas*, why do human outbreaks remain so rare? In this case, the patient reported having eaten the visceral mass. This portion of the animal usually is not included in common gastronomical preparations.

In this paper, 5,6,11-trideoxyTTX was found in an amount 3 times higher than TTX in all samples. With comparison of the concentrations of TTX and 5,6,11-trideoxyTTX obtained by LC−MS to concentrations of both toxins by mouse bioassay, it was demonstrated that the analogue is almost not toxic. This coincides with previous studies where several analogues including 5,6,11-trideoxyTTX are shown as having low toxicity.[3] 5,6,11-TrideoxyTTX can be less toxic because it has less hydroxyls groups compared with the TTX, and for this reason could have less affinity for the binding to the sodium channel. However, this analogue

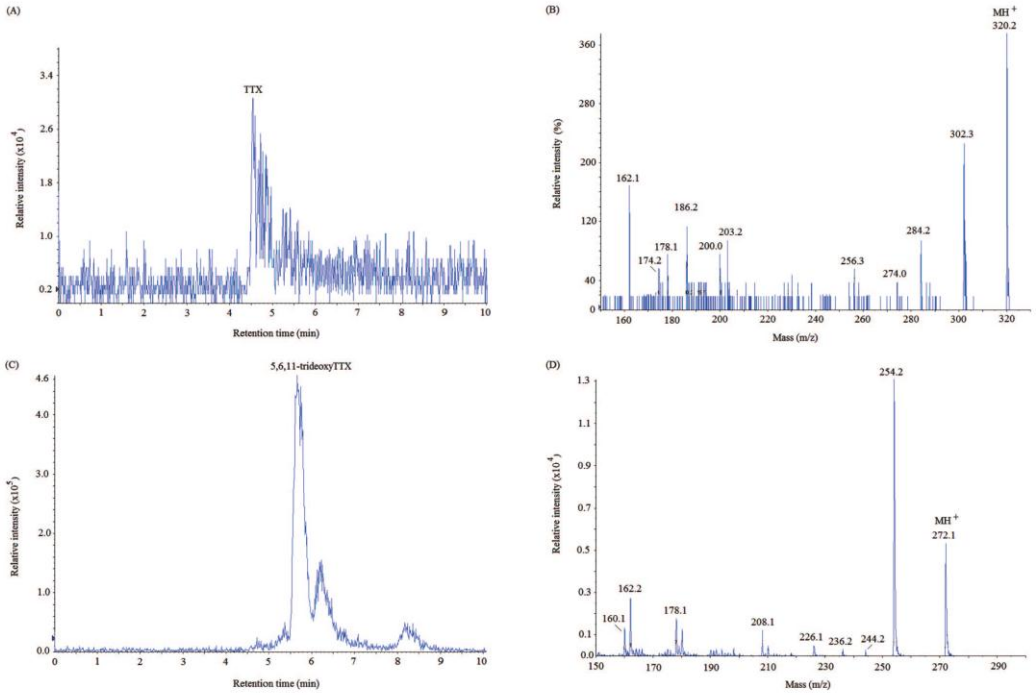

Figure 6. LC−MS/MS analysis in the EPI mode of a urine sample. (A) LC−MS/MS chromatogram for *m/z* 320. (B) LC−MS/MS mass spectrum from part A. (C) LC−MS/MS chromatogram for *m/z* 272. (D) LC−MS/MS mass spectrum from part C.

Table 1. Analysis by LC−MS

	TTX level	5,6,11-trideoxyTTX
sample 1 (digestive gland)	31.5 mg/100 g	100.4 mg/100 g
urine	211.1 ng/mL	692.1 ng/mL
serum	26.4 ng/mL[a]	86.5 ng/mL[a]

[a] Concentrations just at the limit of detection.

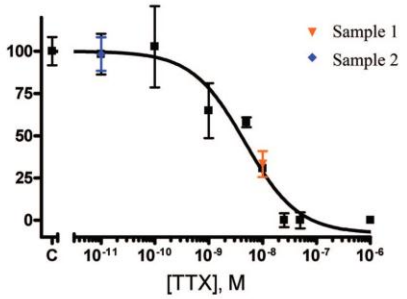

Figure 7. Confocal microscopy analysis of sample 1 (digestive gland) and sample 2 (meat) corresponding to the trumpet shell *Charonia lampas lampas*.

involves other complex changes including dehydrogenation of the C-10-hydroxyl, which is believed to be essential for the affinity,[2] competing with TTX on intracellular targets.

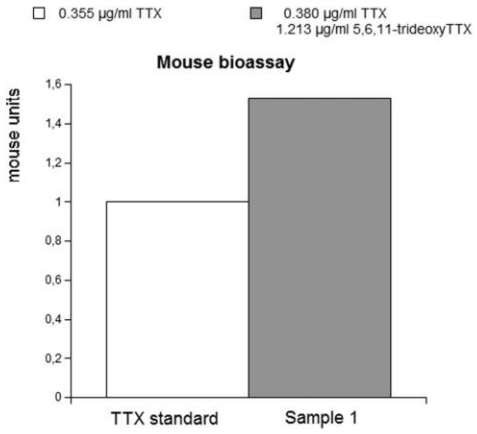

Figure 8. Mouse toxicity assay. TTX standard (Calbiochem Corporation) and extract of digestive gland (sample 1) injected i.p. into mice (19−23 g body weight).

This paper describes the analytical results of a TTX intoxication, from an instrumental, toxicological, and kinetic viewpoint. The most striking observation is the fact that a new, nonregulated toxin has appeared in gastropods in Europe. This is clearly a matter for concern, since this new phenomenon is probably to be attributed to a potential ecological change due to increased

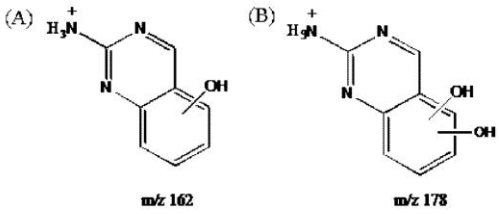

Figure 9. Fragment ions at *m*/*z* 162 (A) and *m*/*z* 178 (B) of MH^+ of TTX and 5,6,11-trideoxyTTX.

warm temperatures. This could pose a hint of potential consequences of global warming on food safety.

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I.2: Intoxicación alimentaria por tetrodotoxina: Primer caso en Europa.

Resumen

La TTX es considerada la toxina más letal en el medio marino. Previamente se han descrito casos de intoxicación correspondientes al consumo de TTX en las regiones tropicales y subtropicales de Asia o en las islas del Pacífico. En esta publicación se presenta el primer caso europeo de intoxicación por TTX en un paciente que ingirió parte de una caracola (*Charonia lampas*) procedente del océano Atlántico en el sur de Europa. Informe del caso: El paciente sufrió una parálisis general, incluyendo los músculos respiratorios, unos minutos después del consumo de unos pocos gramos de la caracola. Se necesitó intubación y ventilación mecánica durante 52 horas después de la intoxicación. Los correspondientes estudios electrofisiológicos no mostraron una excitabilidad completa, ni registros de conducción nerviosa o motora. Se ha detectado la presencia de TTX en el molusco y en la sangre y orina del paciente por medio de la técnica analítica LC-MS. Además, un análisis por MBA mostró cantidades extremadamente altas de toxina en el molusco. Por lo tanto, se concluyó que pueden aparecer biotoxinas muy perjudiciales en las costas europeas, poco habituales en estas latitudes. Este caso debería ser incluido en el diagnóstico diferencial de casos similares en Europa y a partir de ahora deberíamos de estar alerta de la posible presencia de estas toxinas en Europa.

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Selected Topics: Toxicology

SEAFOOD INTOXICATION BY TETRODOTOXIN: FIRST CASE IN EUROPE

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□ **Abstract—Background: Tetrodotoxin is considered the most lethal toxin in the marine environment. Prior cases of intoxication previously described correspond to consumption of tetrodotoxin in tropical or subtropical regions of Asia or the Pacific Islands. Objectives: We present the first European case of tetrodotoxin intoxication in a patient who ingested part of a trumpet shellfish (*Charonia sauliae*) from the Atlantic Ocean in Southern Europe. Case Report: Our patient suffered general paralysis, including the respiratory muscles, a few minutes after the consumption of a few grams of *C. sauliae*. Intubation and mechanical ventilation were necessary for 52 h after the intoxication. The corresponding electrophysiologic studies showed complete non-excitability, with no recordable sensory or motor nerve conduction. We detected the presence of tetrodotoxin in the mollusk and the patient's blood and urine by means of high-performance liquid chromatography-mass spectrometry analysis technique. A previous bioassay showed extremely high quantities of the toxin in the mollusk. Conclusions: This case alerts us to the possibility of a very harmful biotoxin in European coastal waters. This now should be included in the differential diagnosis of similar cases in Europe, and we must be vigilant for its possible presence in Europe.** © 2010 Elsevier Inc.

□ **Keywords—tetrodotoxin; poisoning; respiratory failure; seafood intoxication**

INTRODUCTION

Biotoxins are substances synthesized by living organisms that are harmful to humans. They can be produced by certain bacteria, fungi, vertebrates, or marine microorganisms (unicellular algae that usually live in symbiosis with tropical and subtropical waters in reefs) (1). These microalgae are able to synthesize a large group of toxins whose clinical action in humans affects the gastrointestinal tract and the peripheral nervous system to different degrees, depending on the biotoxin. They have a chemical structure consisting of a common quinazolinic derivative with different associated ions. The toxin levels increase by bioaccumulation along the food chain from the microalgae to the human (2).

Tetrodotoxin (TTX) is a toxin that is probably produced by microalgae, although the class of microalga has yet to be identified. It is considered the most lethal toxin coming from the marine environment (3). It is named after the Tetraodontidae family of fish, although it was later found in other fish, mollusks, and even in amphibians and land animals such as the atelopid frog of Costa Rica, or newts (4–6). Fish, as well as the other carrier animals, are unaffected by the toxins (7). It especially concentrates in the skin and gonads of fish, amphibians, or reptiles, and in the intestine and liver of mol-

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lusks. These organs can accumulate large amounts of heavy metals or other ingested toxic substances. The trumpet shellfish (*Charonia lampas sauliae*) already has been implicated in some of the very few cases of TTX intoxication (8,9).

We present the case of a patient who ingested *C. sauliae* that was contaminated by large concentrations of TTX. The fish was caught off the southern coast of Europe.

CASE REPORT

The patient was a 49-year-old man, a moderate alcohol drinker and cigarette smoker, with no significant past medical history. At a fish market in Malaga, Spain, he bought a *C. sauliae* for personal consumption (Figure 1). The mollusk was caught off the southern coast of Portugal, landed in Huelva (southern Spain), and was transported in a refrigerated truck. Immediately before consumption, it was boiled for 45 min. A few minutes after eating the ventral portion of the *C. sauliae,* the patient started feeling perioral numbness, which extended to both arms, followed by abdominal pain, nausea, and vomiting. He then felt an immediate decrease in strength, starting distally and spreading proximally. When he was transferred by the Emergency Services ambulance, he was conscious, with general muscle weakness, but normal vital signs. He was admitted to the Emergency Department of our hospital, where he required immediate endotracheal intubation and mechanical ventilation due to difficulty breathing. The patient remained conscious until the intubation, with stable vital signs.

Computed tomography (CT) scan and magnetic resonance imaging (MRI) of the skull showed the supratentorial areas and brainstem to be normal. The electrocardiogram showed a normal rhythm and a rate of 95 beats/min. Laboratory results, including liver and renal function tests, a hemogram, coagulation, and cerebrospinal fluid examination, were all normal. A screening of the urine for toxins, including opioids, cocaine, cannabis, amphetamines, benzodiazepines, and barbiturates, was also negative.

Figure 1. Remains of Charonia sauliae consumed by the patient.

The patient was admitted to our Intensive Care Unit (ICU). The only sedatives given were midazolam 10 mg along with succinylcholine 80 mg for the intubation. A propofol and remifentanile perfusion was begun while the patient was connected to the ventilator. The patient continued to show a lack of motor response, non-reactive pupils, absent corneal and oculomotor reflexes, and abolition of deep tendon reflexes for the first 25 h. The heart rate and blood pressure were normal. Twenty-five hours after eating the shellfish, he started showing minimal motor activity, with a completely normal level of consciousness during the following hours. He remained quadriplegic for another 6 h, after which he started moving the neck muscles, progressively recovering enough strength to be extubated 30 h after intubation. The patient was discharged from the ICU 72 h after admission, having recovered normal levels of strength, reflexes, and sensation.

Neurophysiologic studies included an electroencephalogram (EEG) 24 h after toxin ingestion, which was normal. Sensory nerve and motor conduction studies at the same time showed complete non-excitability, with no recordable conductions. Forty-eight hours after consumption, they started improving (Figure 2).

The non-ingested part of the shellfish was stored in a freezer at −20°C. Initially, a mollusk bioassay was carried out to determine the biotoxins in the shellfish (10). The results showed 151 μg and 25,500 μg of toxin/100 g in the mollusk's meat and digestive glands, respectively (the limit allowed by Spanish legislation is 80 μg/100 g). The remaining part of the mollusk and the patient's urine and blood drawn 32 h after consumption were sent to the Department of Pharmacology at the University of Santiago de Compostella, Galicia, Spain, for optimum identification of the toxin. High-performance liquid chromatography (HPLC) studies with the Lawrence method to determine the presence of saxitoxin found none (11). Due to the symptoms reported, the sample was studied for the presence of tetrodotoxin. An HPLC analysis was carried out. The mass spectrometer was operated in enhanced mass spectrum mode to confirm the presence of TTX, and in enhanced product ion (EPI) mode to quantify the toxin.

Urine and blood samples collected from the patient were frozen at −20°C. A sample of *C. sauliae* digestive gland was homogenized and extracted according to the official procedure for paralytic shellfish poisoning (PSP). The sample cleanup procedure used was that

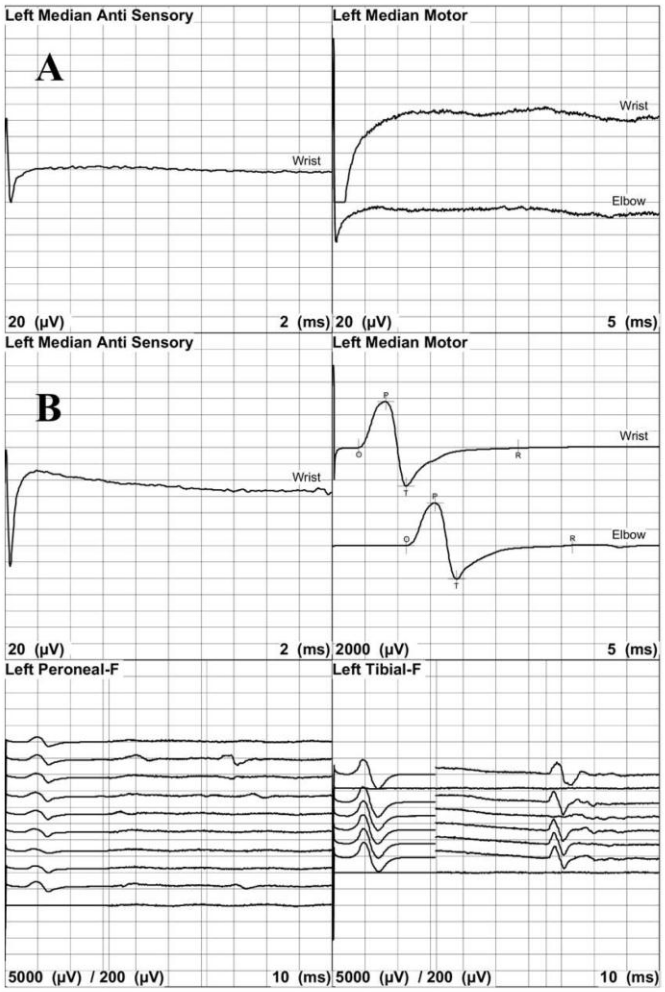

Figure 2. Results of neurophysiologic studies. (A) Sensory and motor conduction studies 24 h after toxin ingestion. Electroneurography and electromyography (EMG) show profound nerve inexcitability, with no recordable sensory or motor nerve conduction. No EMG activity (neither spontaneous or voluntary or reflex activity) was detected. (B) Sensory and motor conduction studies and F-wave responses 48 h after toxin ingestion. Nerve conductions were of a high threshold, no sensory conductions were recordable, and motor nerve studies had a slow conduction, reduced amplitude compound potentials, prolonged distal latencies, and reduced conduction velocities. F-wave latencies were either absent or prolonged. Repetitive studies with slow and high frequencies (ulnar nerve) were normal.

previously developed for TTX and saxitoxin biological samples (12).

Figure 3 shows the HPLC chromatogram of the TTX standard and samples of the *C. sauliae*, urine, and blood analyzed in the EPI mode. These results were transformed into the amount of TTX by using the calibration curve. The level of TTX in the *C. sauliae* was 24.85 mg/100 g of digestive gland, and in the patient's urine and blood sample it was 285.38 ng/mL and 24.54 ng/mL, respectively. The presence of TTX in the mollusk and the patient's blood and urine was therefore confirmed.

DISCUSSION

Previously reported cases of TTX intoxication have occurred in the tropical and subtropical regions of Asia and the Pacific (13–16). Only a very few cases have been

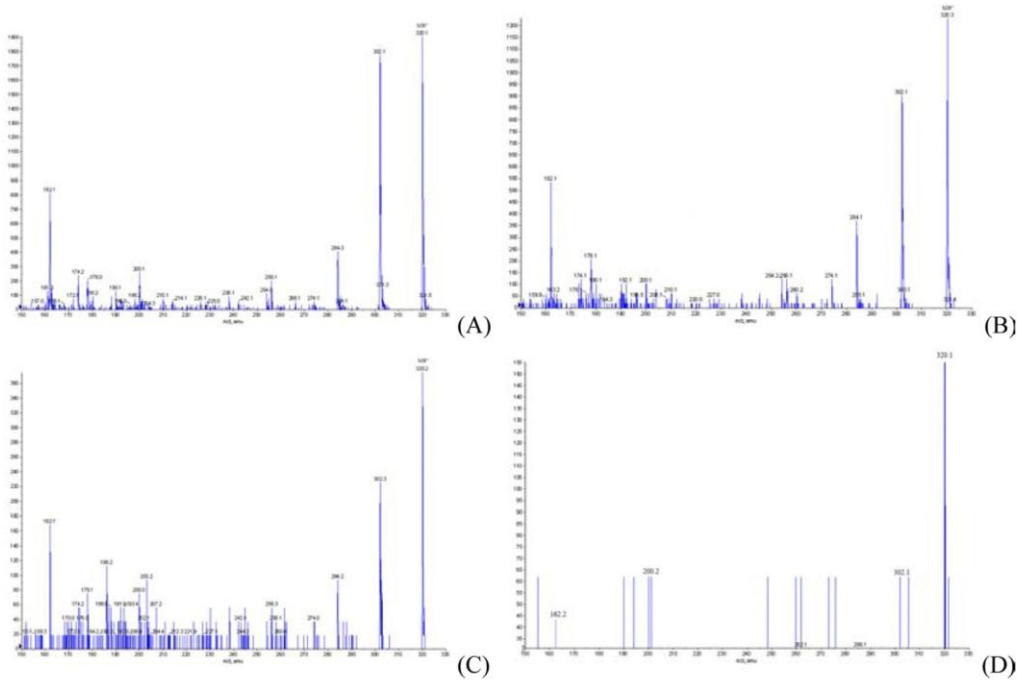

Figure 3. Analysis of tetrodotoxin (TTX), *Charonia sauliae*, and patient samples. Liquid chromatography-mass spectrometry (LC-MS) analysis of TTX standard, *Charonia sauliae*, and patient samples. LC-MS-MS mass spectra of (A) TTX, (B) *C. sauliae* sample, (C) urine sample, and (D) blood sample.

described in the United States, which were caused by fish imported from Japan or Taiwan (17). The *C. sauliae* ingested by our patient was caught off the southern Portuguese coast during September 2007, which is when the waters are warmest. This case is of great epidemiologic interest, as it is the first reported intoxication by TTX from fish caught off the European coast. This finding alerts us to the possibility that the microalgae responsible have now reached the Atlantic Ocean, and are spreading through less historically warm areas. Just as population migration is bringing diseases that were formerly associated with tropical areas to developed countries, the marine environment is experiencing worldwide temperature changes that may result in the transmission of diseases to new areas. Their detection by emergency physicians will be the first step to alert health authorities to the need for possible preventive measures.

Saxitoxin is the most common toxin produced by marine microalgae in colder waters, that is, those above 30° latitude north and below 30° latitude south (18). Our initial study was therefore directed at determining the presence of this toxin once the bioassay test for PSP was positive. After seeing that it was negative for saxitoxin, we then sought TTX. Saxitoxin is very similar structurally to TTX; they are both thermostable, and have a common mechanism of action, with intoxication causing superimposable symptoms. They are normally ingested via bivalve shellfish, for example, mussels or clams, that have filtered toxic microalgae, sometimes causing red tides. The two intoxications can be differentiated only by their area of distribution or by isolation and identification of the toxin using the Lawrence method (11).

The consumption of the digestive glands of the mollusk, about 5 g, was enough to produce the symptoms described. The severity of the clinical effects suggests the involvement of a very high concentration of TTX. Its consumption is associated with a mortality rate that can reach 60% without treatment (19). The intensity and speed of the symptoms depend on the toxin's concentration. In our case, the onset and severity of the symptoms corresponded to a maximum degree of TTX intoxication (20). Even though the shellfish was boiled for 45 min just before consumption, this failed to reduce its toxicity, as TTX is heat-stable and not dam-

aged by heating or freezing (21). TTX has no particular odor, color, or taste.

TTX binds to the sodium channel of the nerve, preventing the passage of sodium ions through the cell membrane, thereby blocking the passage of the nerve impulse (22,23). The transient and quickly reversible nerve dysfunction caused by ion channel blockade in our patient is typical. Although there was a complete lack of neurological response, the EEG was normal. Our patient was hemodynamically stable throughout and showed no cardiac dysrhythmia, though rhythm alterations have been described through the blockade of the conduction and excitability of the myocardial tissue. We believe that cardiac dysrhythmia in other cases may have been caused by hypoxia secondary to respiratory muscle paralysis.

The initial clinical diagnosis was made due to the sudden appearance of symptoms after ingesting shellfish. The short latency period and the meal being boiled excluded intoxication by *clostridium botulinum*, which has a thermosensitive toxin, a different mechanism of action, and a specific treatment. Guillain-Barré syndrome was also ruled out due to the rapid onset of symptoms. A cerebrovascular accident in the brainstem was excluded by CT scan and MRI. A urine study for other possible toxins was also negative. Diagnostic confirmation requires analysis of samples of the fish and the patient's blood and urine (24).

We initially performed a bioassay. This technique is not specific for the type of toxin and has poor sensitivity. A second analysis was therefore carried out with high-performance liquid chromatography. This is a well-known and trusted technique to detect and quantify toxins in any biological sample (25). The first option was to look for the presence of saxitoxin and its analogs. The biotoxins determined by bioassay are PSP, and the results expressed as the equivalent amount of saxitoxin/100 g. However, HPLC analysis of the extracts showed no saxitoxin. As the symptoms are similar, we then sought TTX using liquid chromatography-mass spectrometry (LC-MS). With this technique, high amounts of TTX were detected in both the *C. sauliae* samples and the patient's fluids. Similar results and technology have been reported for other human TTX intoxication after fish consumption (12).

No antidote is available after toxin ingestion. Treatment is supportive (26). When intoxication is severe, as in our case, intubation and mechanical ventilation are required. It is important to sedate patients when they are intubated, as mentation can be normal. Supportive care beyond 48 h isn't usually necessary because the toxin is eliminated from the blood, becoming undetectable after 24 h (25). We extracted blood and urine samples 30 h after consumption, showing just enough serum concentration to be detected, although high toxin concentrations were still present in the urine. The prognosis can be considered excellent if there are no complications deriving from ventilatory and cardiovascular support.

CONCLUSION

In conclusion, this case alerts us to the possibility of a very harmful toxin from fish entering our coastal waters. Its presence, therefore, should be considered in the differential diagnosis of paralysis after seafood ingestion, and the health authorities warned to find means to detect and possibly prevent its entrance onto the market.

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REFERENCES

1. Salzman M, Madsen JM, Greensberg MI. Toxins: bacterial and marine toxins. Clin Chem Lab Med 2006;26:397–419.
2. Lewis RJ, Holmes MJ. Origin and transfer of toxins involved in ciguatera. Comp Biochem Physiol C 1993;106:615–28.
3. Halstead BW. Dangerous marine animals: that bite-sting-shock-are non-edible. Cambridge, MD: Cornell Maritime Press; 1959.
4. Yin HL, Lin HS, Huang CC, et al. Tetrodotoxication with nassauris glans: a possibility of tetrodotoxin spreading in marine products near Pratas Island. Am J Trop Med Hyg 2005;73:985–90.
5. Kim YH, Brown GB, Mosher HS, Fuhrman FA. Tetrodotoxin: occurrence in Atelopid frogs of Costa Rica. Science 1975;189:151–2.
6. Mosher HS, Fuhrman FA, Buchwald HD, Fischer HG. Tarichatoxin-tetrodotoxin: a potent neurotoxin. Science 1964;144:1100–10.
7. Helfrich P, Banner AH. Experimental induction of ciguatera toxicity in fish through diet. Nature 1963;197:1026–7.
8. Narita H, Noguchi T, Maruyama J, et al. Occurrence of tetrodotoxin on a trumpet shellfish "boshubora" *Charonia sauliae*. Nippon Suisan Gakkai Shi 1981;47:934–41.
9. Narita H, Matsubara S, Miwa N, et al. *Vibrio alginolyticus*, a TTX-producing bacterium isolated from the starfish *Astropecten polyacanthus*. Nippon Suisan Gakkai Shi 1987;53:617–21.
10. McFarren EF. Report of collaborative studies of the bioassays for paralytic shellfish poison. J Assoc Off Anal Chem 1959;42:263–71.
11. Lawrence JF, Wong B, Menard C. Determination of decarbamoyl saxitoxin and its analogues in shellfish by prechromatographic oxidation and liquid chromatography with fluorescence detection. J AOAC Int 1996;79:1111–5.
12. Tsai YH, Hwang DF, Cheng CA, Hwang CC, Deng JF. Determination of tetrodotoxin in human urine and blood using C18 cartridge column, ultrafiltration and LC-MS. J Chromatogr B Analyt Technol Biomed Life Sci 2006;832:75–80.
13. Ahasan HA, Mamun AA, Karim SR, et al. Paralytic complications of puffer fish (tetrodotoxin) poisoning. Singapore Med J 2004;45:73–4.
14. Ellis RM, Jelinek GA. Never eat an ugly fish: three cases of tetrodotoxin poisoning from Western Australia. Emerg Med 1997;9:136–42.
15. Kanchanapongkul J. Puffer fish poisoning: clinical features and management experience in 25 cases. J Med Assoc Thai 2001;84:385–9.

16. Trevett AJ, Mavo B, Warrell DA. Tetrodotoxin poisoning from ingestion of a porcupine fish (Diodon hystrix) in Papua New Guinea: nerve conduction studies. Am J Trop Med Hyg 1997;56:30–2.
17. Centers for Disease Control and Prevention. Tetrodotoxin poisoning associated with eating puffer fish transported from Japan—California, 1996. MMWR Morb Mortal Wkly Rep 1996;45:389–91.
18. Lehane L. Paralytic shellfish poisoning: a potential public health problem. Med J Aust 2001;175:343–4.
19. Ellenhorn MJ, Barceloux DG. Medical toxicology: diagnosis and treatment of human poisoning. New York: Elsevier Science Publishing Company, Inc.; 1988.
20. Isbister GK, Son J, Wang F, et al. Puffer fish poisoning: a potentially life-threatening condition. Med J Aust 2002;177:650–3.
21. Sorokin M. Puffer fish poisoning. Med J Aust 1973;1:73–4.
22. Cestele S, Catterall WA. Molecular mechanisms of neurotoxin action on voltage-gated sodium channels. Biochimie 2000;82:883–92.
23. Kao CY. Tetrodotoxin, saxitoxin and their significance in the study of excitation phenomena. Pharmacol Rev 1966;18:997–1049.
24. O'Leary MA, Schneider JJ, Isbister GK. Use of high performance liquid chromatography to measure tetrodotoxin in serum and urine of poisoned patients. Toxicon 2004;44:549–53.
25. Holland PT. Analysis of marine toxins—techniques, method validation, calibration standards and screening methods. In: Botana LM, ed. Seafood and freshwater toxins: pharmacology, physiology, and detection, 2nd edn. New York: CRC Press; 2008:21–50.
26. Lawrence DT, Dobmeier SG, Bechtel LK, Holstege CP. Food poisoning. Emerg Med Clin North Am 2007;25:357–73.

I.3: Método de cromatografía líquida con espectrometría de masas para detectar tetrodotoxina y sus análogos en peces globo *Lagocephalus sceleratus* (Gmelin, 1789) capturados en aguas Europeas.

Resumen

La presencia de la TTX y sus análogos, 4-*epi*TTX, 4,9-anhydroTTX, 5-deoxyTTX, 11-deoxyTTX, 5,6,11-trideoxyTTX, 11-norTTX-6 (S)-ol y 11 -norTTX-6 (R)-ol se investigó por primera vez en cinco tejidos diferentes (hígado, gónadas, tracto gastrointestinal, músculo y piel) de seis ejemplares del pez globo de la especie *Lagocephalus sceleratus* procedentes de aguas europeas (Mar Egeo, Grecia) mediante el uso de la LC-MS de ionización por electrospray (ESI), operando en el modo convencional de baja energía de disociación inducida por colisión (CID) de MS/MS. Dos isómeros de la 5,6,11-trideoxyTTX se detectaron en todas las muestras como los principales análogos de la TTX, seguido por el análogo 11-deoxyTTX, 11-norTTX-6(S)-ol y la TTX. Además, pequeñas cantidades de 4-*epi*TTX, 4,9-anhydroTTX, 5-deoxyTTX y 11-norTTX-6(R)-ol, se encontraron en la mayoría de los tejidos analizados. En todas las muestras de pez globo, se detectaron mayores niveles de toxina en las gónadas, el tracto gastrointestinal y el hígado, mientras que en el músculo y la piel las cantidades fueron más bajas. La distribución de la toxina en los tejidos de las muestras de los seis ejemplares de *L. sceleratus* fue diferente en función del tamaño de los peces, la zona y la temporada donde estos fueron capturados. El método de LC-ESI-CID-MS/MS utilizado en esta publicación se propone como una técnica adecuada para la determinación de TTX y sus análogos, con un bajo límite de detección (0,08 µg/g).

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Analytical Methods

Liquid chromatography–mass spectrometry method to detect Tetrodotoxin and Its analogues in the puffer fish *Lagocephalus sceleratus* (Gmelin, 1789) from European waters

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ABSTRACT

The presence of tetrodotoxin (TTX) and its analogues, 4-*epi*TTX, 4,9-anhydroTTX, 5-deoxyTTX, 11-deoxyTTX, 5,6,11-trideoxyTTX, 11-norTTX-6(S)-ol, and 11-norTTX-6(R)-ol was investigated for the first time in five different tissues (liver, gonads, gastrointestinal tract, muscle, and skin) of six specimens of the marine puffer fish *Lagocephalus sceleratus* from European waters (Aegean Sea, Greece) by using liquid chromatography coupled to electrospray ionisation mass spectrometry operating in the conventional mode in addition to low-energy collision dissociation tandem mass spectrometry (CID-MS/MS).

Two isomers of 5,6,11-trideoxyTTX were detected in all specimens as the major TTX analogues, followed by 11-deoxyTTX, 11-norTTX-6(S)-ol, and TTX. However, minor amounts of 4-*epi*TTX, 4,9-anhydroTTX, 5-deoxyTTX, and 11-norTTX-6(R)-ol were also found in most of the tested tissues. In all puffer fish specimens, gonads, gastrointestinal tract, and liver contained the highest toxin levels, whereas muscle and skin contained lower amounts. Toxin distribution within the tissues of the six *L. sceleratus* specimens was different depending on fish size, area, and season where fish were caught. The LC-ESI-CID-MS/MS analysis employed is proposed as a suitable technique for determination of TTX and its analogues with a low detection limit (0.08 μg/g).

1. Introduction

Tetrodotoxin (TTX), one of the most potent neurotoxin was thought to occur only in puffer fish. However, since 1965, distribution of TTX and its analogues has been spread over a variety of animals (Miyazawa & Noguchi, 2001). As a consequence of the opening of the Suez Canal, invasive species originated from the Red sea were introduced into the Eastern Mediterranean creating new colonies and becoming a major component of the Mediterranean ecosystem (Galil & Zenetos, 2002). These marine species were termed as Lessepsian migrants (Spanier & Galil, 1991). Nowadays, more than 300 species native to the Red Sea have been identified in the Mediterranean Sea. *Lagocephalus sceleratus* (Gmelin, 1789) originating from the Indo-Pacific region is a puffer fish species of the tetraodontidae family. It was reported for the first time in the Mediterranean region in Gökova Bay (Southeastern Aegean Sea, Turkey) on February 2003 and on November 2004 in the Israeli coast (Akyol, Ünal, Ceyhan, & Bilecenoglu, 2005; Golani & Levy, 2005). Later on, it was recorded in 2005 in the Cretan Sea (Aegean, Greece) (Kasapidis, Peristeraki, Tserpes, & Magoulas, 2007). Since then the occurrence of *L. sceleratus* in the Rhodes Island shows that its population is continuing to extend in the Southeast Aegean Sea. The associated risk of food poisoning by accidental capture and consumption of this species, commonly containing TTX, has increased in Greek waters (Katikou, Georgantelis, Sinouris, Petsi, & Fotaras, 2009).

In marine species of puffer fish, liver and ovary generally show the highest toxicity, followed by intestines and skin. Ingestion of puffers that exhibited the highest toxicity in the skin has occasionally caused food poisoning with serious cases in Thailand and Bangladesh (Laobhripatr, et al., 1990; Mahmud, Arakawa, & Noguchi, 2000). However the musculature can also be toxic in some species, such as *Lagocephalus lunaris* and *Chelonodon patoca* (Noguchi, Arakawa, & Takatani, 2006). Recent incidents after consumption of *L. sceleratus* were presented between 2005 and 2008 on the Israeli coast of the eastern Mediterranean. The most severely poisoned patient ate the entire liver of the fish (Bentur, et al., 2008).

In toxic puffer fish, TTX can co-exist with various of its analogues (TTXs, Fig. 1). Among them, 4-*epi*TTX and 4,9-anhydroTTX are the chemical equilibrium analogues of TTX (Goto, Kishi, Takahashi, & Hirata, 1965; Nakamura & Yasumoto, 1985). Four deoxy analogues, such as, 5-deoxyTTX (Yotsu-Yamashita, Schimmele, &

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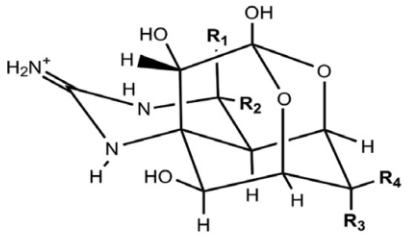
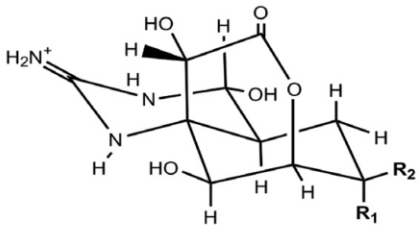
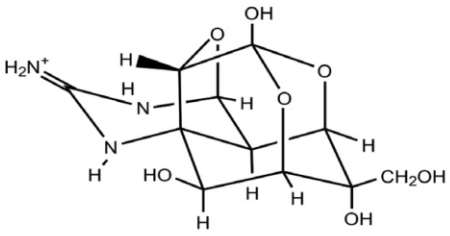


	R1	R2	R3	R4	MH^+ (m/z)
TTX	H	OH	OH	CH_2OH	320
4-*epi*TTX	OH	H	OH	CH_2OH	320
11-deoxyTTX	H	OH	OH	CH_3	304
11-norTTX-6(S)-ol	H	OH	OH	H	290
11-norTTX-6(R)-ol	H	OH	H	OH	290



	R1	R2	MH^+ (m/z)
5-deoxyTTX	OH	CH_2OH	304
5,6,11-trideoxyTTX	H	CH_3	272



4,9-anhydroTTX MH^+ *m/z* 302

Fig. 1. Chemical structures of TTXs with *m/z* for MH^+ ions.

Yasumoto, 1999), 11-deoxyTTX (Yasumoto, Yotsu, Murata, & Naoki, 1988), 6,11-dideoxyTTX (Jang & Yotsu-Yamashita, 2007), 5,6,11-trideoxyTTX (Yotsu-Yamashita, Yamagishi, & Yasumoto, 1995), and two 11-nor analogues, 11-norTTX-6(R)-ol (Endo, Khora, Murata, & Yasumoto, 1988) and 11-norTTX-6(S)-ol (Yotsu, Hayashi, Khora, Sato, & Yasumoto, 1992) have also been isolated from puffer fish and newts as the chemical non-equilibrium analogues. Bacterial production of TTX has been reported (Noguchi, et al., 1986; Yasumoto, et al., 1986), but it has not been clarified whether these chemically non-equilibrium analogues are also produced by the TTX-producing organisms, or if transformation occurs within the puffer fish organism (Kono, et al., 2008).

Several studies on the toxicity of puffer fish, particularly *L. sceleratus*, were carried out in the Gulf of Suez and Red Sea by other authors (Sabrah, El-Ganainy, & Zaky, 2006; Sherif, Ali, Abbas, & Mohamed, 1994; Zaki, 2004). However, determination of TTX and its analogues by liquid chromatography coupled with mass spectrometry (LC–MS) in this fish species has not yet been widely investigated.

In this manuscript, we examine for first time the use of single stage LC-ESI-MS in addition of making use of the stage-of-the-art low-energy collision dissociation tandem mass spectrometric (CID-MS/MS) analysis using the multiple reaction monitoring (MRM) mode to quantify the content of TTX and its analogues. These included the 4-*epi*TTX, 4,9-anhydroTTX, 5-deoxyTTX, 11-deoxyTTX, two isomers of 5,6,11-trideoxyTTX, 11-norTTX-6(S)-ol, and 11-norTTX-6(R)-ol. All of the TTX and derived compounds were present in the different tissues of six specimens of the puffer fish *L. sceleratus* collected in the Aegean Sea, some of which were positive for TTX in previous biological assays (Katikou, et al., 2009).

2. Material and methods

2.1. Chemicals

Solvents used were either HPLC or analytical grade, whereas water was distilled and passed through a water purification system Arium 611 from Sartorius (Germany). Methanol, acetonitrile, and ammonium hydroxide were purchased from Panreac Quimica S.A. (Spain). Ammonium formate, trimethylamine (TMA, 25% solution in water), and heptafluorobutyric acid were from Sigma

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Aldrich (Spain). Formic acid was obtained from Merck (Spain). The Tetrodotoxin (TTX) used as a calibrant was from Calbiochem Corporation. Standard solutions of TTX were dilutions of the stock solution with methanol.

2.2. Puffer fishes specimens

Specimens No. 1, 2, 3, 4, and 6 of the puffer fish *L. sceleratus* were collected by trawl fishing in the Southeast Aegean Sea, near the island of Rhodes, in depths between 27 and 36 m during October–December 2007. Specimen No. 5 was caught in the North West part of Aegean Sea in the area of Horeyto, Pelion in June 2007. Specimens No. 1, 2, and 3 are corresponding with groups of 15, 14, and 11 small fishes, respectively and the specimens No. 4, 5, and 6 with a single large fish. All the samples were immediately frozen and transferred to the National Reference Laboratory on Marine Biotoxins (NRLMB, Institute of Food Hygiene, Thessaloniki, Greece) and there were kept frozen at −70 °C until sample preparation and toxin extraction.

2.3. Preparation of sample solutions

Specimens (individual or group of fishes) were initially dissected into the muscle, liver, skin, gastrointestinal tract, and gonads (where available; specimens No. 4, 5, and 6). Toxin extraction from each tissue was carried out in acid medium (0.1% acetic acid) by heating in a boiling water bath following a procedure previously described (Katikou, et al., 2009). Acidic tissue extracts were then evaporated, and the residue was dissolved in 0.5 ml of methanol, and then filtered through 0.45 μm filters (Ultrafree-MC centrifugal filter devices from Millipore, Spain). The filtrate was used as the sample solution for LC–MS analysis.

2.4. Liquid chromatography-electrospray ionisation tandem mass

The high-performance liquid chromatography (LC) equipment was formed by a binary system of LC-10ADVP pumps, an autoinjector (SIL-10ADvp) with refrigerated rack, degasser (DGU-14A), column oven (CTO-10ACvp), and the system controller (SCL-10Avp) from Shimadzu (Japan). This system is coupled to a 2000 QTRAPLC/MS/MS instrument from Applied Biosystems (USA), which consists of a hybrid quadrupole-linear ion trap mass spectrometer (MS) equipped with an atmospheric pressured ionisation (API) fitted with an electrospray ionisation source (ESI). Nitrogen was produced by a Nitrocraft $NC_{LC/MS}$ generator from Air Liquide (Spain).

2.5. LC–MS conditions

The LC was operated with the ESI interface in the positive ion mode using the following parameters: curtain gas, 15 psi; collision-activated dissociation gas, 6 psi; IonSpray voltage, 4000 V; temperature, 450–500 °C; gas 1, 50 psi; gas 2, 50–75 psi; these parameters had been previously optimised using the TTX toxin standard. Analyst software was used for instrument control as well as data processing and analysis.

Several columns were tested to achieve separation of TTX and analogues present in the samples. Column oven temperature was set at 20–25 °C and injection volume was 5 μl. The toxins were first eluted in a Zorbax 300SB-C3 column (i.d. 4.6 × 150 mm; 5 μm) with a guard cartridge (i.d. 4.6 × 12.5 mm) from Agilent Technologies (USA). Then, Sunfire C18 column (i.d. 4.6 × 250 mm; 5 μm) with a guard cartridge (i.d. 4.6 × 10 mm) and XBridge™ Amide column (i.d. 2.1 × 150 mm; 3.5 μm) with a guard cartridge (i.d. 2.1 × 10 mm) from Waters (Spain) were used to elute the toxins. In this case, the mass spectrometer was operated in multiple reaction monitoring (MRM), analysing two product ions per compound: one for quantification and the other for confirmation.

Five protonated molecules [M + H]+ at *m/z* 320, 302, 304, 290, and 272 corresponding to TTXs were detected. The MRM transitions selected were: TTX and 4-*epi*TTX: 320 > 302/162; 4,9-anhydroTTX: 302 > 256/162; 11-deoxyTTX and 5-deoxyTTX: 304 > 286/176; 11-norTTX-6(S)-ol and 11-norTTX-6(R)-ol: 290 > 272/162 and 5,6,11-trideoxyTTX: 272 > 254/162. Quantification was done with the most abundant ion in the fragment spectra: 302 (TTX and 4-*epi*TTX), 162 (4,9-anhydroTTX), 286 (11-deoxyTTX and 5-deoxyTTX), 272 (11-norTTX-6(S)-ol and 11-norTTX-6(R)-ol) and 254 (5,6,11-trideoxyTTX).

2.6. Low-energy CID-MS/MS conditions

The mass spectrometer parameters were adjusted to obtain a signal of maximum intensity and stability. For the MS optimisation, the sample solution was directly infused in the electrospray source at a 10 μl/min flow rate with a syringe pump. The MS was operated in the positive ion mode using the product ion scan with a cone gas, 40 V; capillary voltage, 2.8 kV; source temperature, 120 °C; desolvation temperature, 350 °C; collision energy, 45 eV. Helium and nitrogen were used as collision and drying gases, respectively.

3. Results

In order to confirm the presence of TTXs in different tissues of the *L. sceleratus* specimens, the methanolic extracts were injected directly in the ionisation source of the ESI-MS system (+ ion mode) with a syringe pump at 10 μl/min flow rate. Fig. 2 shows the ESI-MS which indicate the diagnostic protonated molecules of the *m/z* 302 corresponding to 4,9-anhydroTTX (Fig. 2A), *m/z* 304 corresponding to the deoxy analogues, such as 5-deoxyTTX and 11-deoxyTTX (Fig. 2B), *m/z* 290 corresponding to the two 11-nor analogues, 11-norTTX-6(R)-ol and 11-norTTX-6(S)-ol (Fig. 2C) and *m/z* 272 corresponding to 5,6,11-trideoxyTTX (Fig. 2D) present in the gonads from No. 6 specimen. After confirming the presence of these TTXs in the samples, different columns, mobile phases and flow rates were tested to elute and separate the toxins detected in the MS mode. Initially, sample solutions were analysed by LC–MS using a previously described method to detect TTX and 5,6,11-trideoxyTTX with the MS operating in ESI mode (Rodríguez, et al., 2008). Toxins were eluted in a Zorbax 300SB-C3 column (i.d. 4.6 × 150 mm, 5 μm) with a mobile phase composed by a mixture of 1% acetonitrile, 10 mM TMA and 10 mM ammonium formate (pH 4.0 adjusted with formic acid), isocratic flow rate 0.4 ml/min. A peak with a retention time of 4.5 min was present in the gonads from No. 6 specimen (Fig. 3A). This toxin was identified as TTX, which was confirmed by comparing with the TTX standard (Fig. 3B). The mass spectrum of TTX, with the formation of the characteristic daughter ions: 302.2, 284.2, 256.1, 178.1, and 162.1 is shown in Fig. 3C. TTX was detected in all tissues of the six puffer fish specimens. Subsequently, the samples were injected with the ESI-MS to examine the presence of TTXs in a range of *m/z* 270–330. As shown in Fig. 4A, several peaks were detected at different retention times in the gonads of the No. 6 specimen. The peak that eluted at 4.4–4.8 min, gave an ESI-MS shown in Fig. 4B. The ESI-MS showed the presence of four protonated molecules at *m/z* 290, 304, 302, and 320, which corresponded to 11-norTTX-6(R)-ol and 11-norTTX-6(S)-ol (*m/z* 290), 5-deoxyTTX and 11-deoxyTTX (*m/z* 304), 4,9-anhydroTTX (*m/z* 302), 4-*epi*TTX, and TTX (*m/z* 320). While all these compounds that are separated on a HPLC column elute in the same retention time, the analogue 5,6,11-trideoxyTTX (*m/z* 272) was mainly eluted at 5.7 min (Fig. 4C). Therefore

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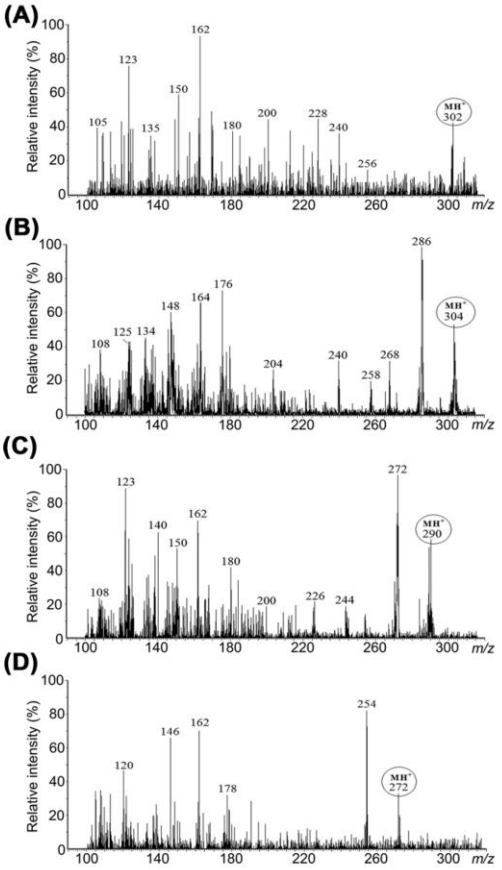

Fig. 2. Fragment ion spectra (MH^+) of (A) 4,9-anhydroTTX (m/z 302); (B) 5-deoxyTTX and 11-deoxyTTX (m/z 304); (C) 11-norTTX-6(S)-ol and 11-norTTX-6(R)-ol (m/z 290) and (D) 5,6,11-trideoxyTTX (m/z 272), present in the gonads of the *L. sceleratus* specimen No. 6. Sample solution was injected into 10 μl/min of methanol. Collision energy: 45 eV.

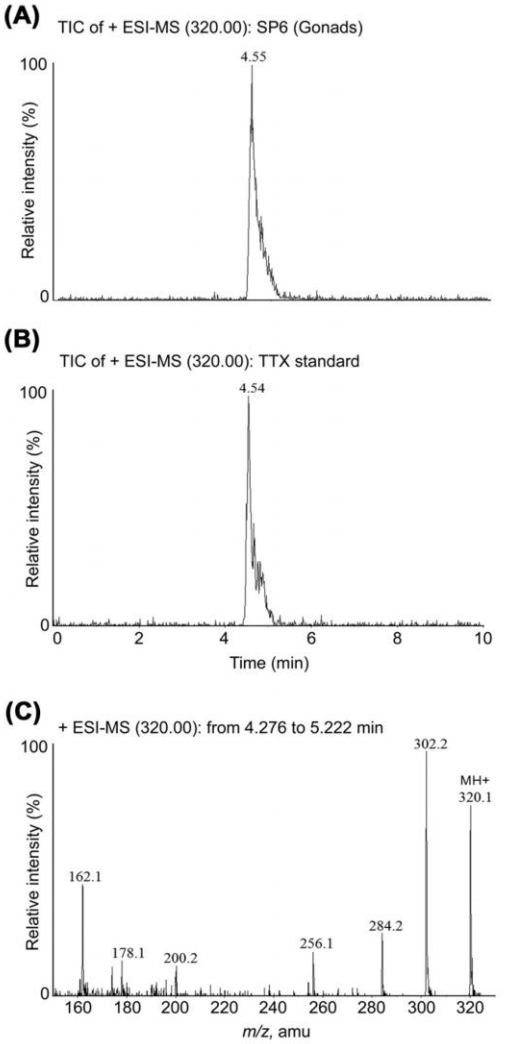

Fig. 3. Mass chromatograms obtained with the MS operating in ESI mode. (A) Gonads from No. 6 specimen of *L. sceleratus*. (B) TTX standard solution (1.5 μg/ml). (C) Fragment ion spectra of TTX from chromatogram B. Toxins eluted in a Zorbax 300SB-C3 column (i.d. 4.6 × 150 mm, 5 μm) at 25 °C, 0.4 ml/min.

with this method (column and mobile phase) it was not possible to separate and distinguish the TTX analogues.

A second method was performed using a Sunfire C18 column (i.d. 4.6 × 250 mm, 5 μm). Toxins were eluted within the column with two mobile phases formed by 1% acetonitrile; 20 mM heptafluorobutyric acid; 20 mM ammonium hydroxide and 10 mM ammonium formate (pH 4.0 with formic acid) (eluent A) and the same mixture but with 5% acetonitrile (eluent B) using the following gradient: 100% A in the first 22 min, 100% mobile phase B for the next 3 min and hold 100% B for 15 min and back to 100% A over the next 3 min before the next injection, flow rate 0.4 ml/min. LC-ESI-CID-MS/MS separation of the TTXs in the gonads from No. 6 specimen is shown in Fig. 5A. The protonated molecules [M + H]+ corresponding to TTX and 4-*epi*TTX (m/z 320), 11-norTTX-6(S)-ol, and 11-norTTX-6(R)-ol (m/z 290); 11-deoxyTTX and 5-deoxyTTX (m/z 304) and 4,9-anhydroTTX (m/z 302) were detected and separated in MRM mode. However 5,6,11-trideoxyTTX (m/z 272) was retained by the column through each injection and could not be detected in any sample.

To overcome this problem, another method was developed using a XBridge Amide column (i.d. 2.1 × 150 mm; 3.5 μm). The LC was operated with eluent A consisting of 10 mM ammonium formate and 10 mM formic acid in water. Eluent B contained 95% acetonitrile and 5% water with a final concentration of 5 mM ammonium formate and 2 mM formic acid. The gradient programme used to elute the toxins was 100% mobile phase B at the beginning and decreasing to 65% B after 15 min, then 65% B kept for 3 min and back to 100% B over the next 2 min and finally kept 100% B for 5 min before the next injection. Flow rate was 0.2 ml/min. Fig. 5B shows the LC-ESI-CID-MS/MS separation of the TTXs in the gonads

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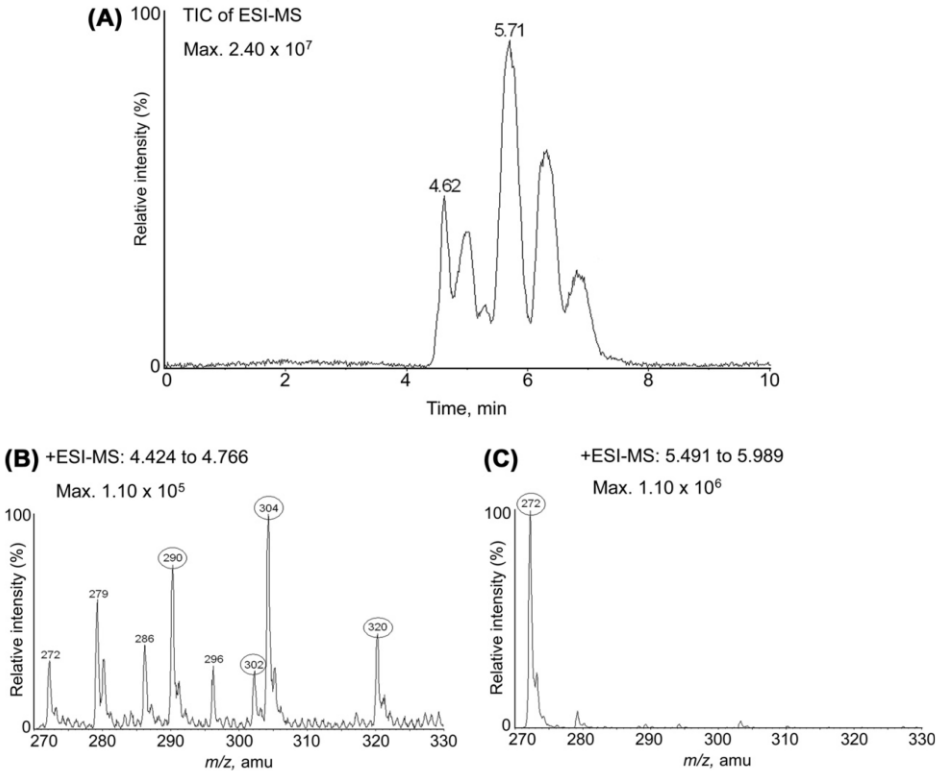

Fig. 4. LC–MS analysis of the gonads from No. 6 specimen of *L. sceleratus*. (A) Full scan TIC chromatogram of the sample obtained with the MS operating in ESI mode at mass range *m/z* 270–330. (B) Mass spectrum of the peak from 4.424 to 4.766 min. (C) Mass spectrum of the peak from 5.491 to 5.989 min. Toxins eluted in a Zorbax 300SB-C3 column (i.d. 4.6 × 150 mm, 5 μm) at 25 °C, 0.4 ml/min.

from No. 6 specimen, again as the most representative sample. In this case two peaks were eluted at 13.08 and 13.56 min identified to contain *m/z* 272 attributed to the 5,6,11-trideoxyTTX (1) and 5,6,11-trideoxyTTX (2). In addition, 5-deoxyTTX (17.49 min), 11-deoxyTTX (18.26 min), 4,9-anhydroTTX (18.35 min), 11-norTTX-6(R)-ol (18.31 min), 11-norTTX-6(S)-ol (19.07 min), 4-*epi*TTX (19.85 min), and TTX (20.53 min) were also identified in the sample in MRM mode with the following transitions: 272 > 254/162 for 5,6,11-trideoxyTTX, 304 > 286/176 for 5-deoxyTTX, and 11-deoxyTTX, 302 > 256/162 for 4,9-anhydroTTX, 290 > 272/162 for 11-norTTX-6(R)-ol, and 11-norTTX-6(S)-ol and 320 > 302/162 for 4-*epi*TTX and TTX. Therefore, a good separation and identification of the toxins was achieved with this method and it was selected to quantify the TTXs presents in the tissues of six *L. sceleratus* specimens. So, a calibration curve was obtained using the TTX standard within the range of 62.5–2000 ng/ml. The limit of detection (LOD) of the assay method for TTX was 16 ng/ml (S/N > 3). The limit of quantification (LOQ; S/N > 10) was reproducible at 63 ng of TTX per ml. As a result, toxins present in the sample were individually quantified based on the peak area on the MRM chromatograms with the curve obtained from TTX standard. TTX (46 μg/g), 4-*epi*TTX (6 μg/g), 4,9-anhydroTTX (5.5 μg/g), 5-deoxyTTX (20 μg/g), 11-deoxyTTX (231 μg/g), 11-norTTX-6(R)-ol (22 μg/g), 11-norTTX-6(S)-ol (222 μg/g), 5,6,11-trideoxyTTX (1) (2.8 mg/g), and 5,6,11-trideoxyTTX (2) (4.8 mg/g) in the gonads of the fish No.6 were estimated. Concentrations obtained for each detected toxin in all tissue extracts of the six specimens of *L. sceleratus* are shown in Table 1. In addition, the results of the previous toxicological analysis by mouse bioassay (MBA) (Katikou, et al., 2009) are shown in Table 1 too. From LC-MS results it can be observed that 5,6,11-trideoxyTTX (1) and 5,6,11-trideoxyTTX (2) were found to be the major TTX analogues in all tissues of the No. 1, 2, 3, 4, and 6 specimens with percentages around 27% and 55%, respectively of the total TTXs present in the samples. 11-deoxyTTX and 11-norTTX-6(S)-ol were detected in minor percentages (8% and 6%, respectively), TTX (3%) and 4-*epi*TTX, 4,9-anhydroTTX, 5-deoxyTTX, and 11-norTTX-6(R)-ol were found at very low rates (1–2%). On contrary, in fish No. 5 tissues, 11-deoxyTTX, 11-norTTX-6(S)-ol, and 11-norTTX-6(R)-ol were detected in higher amounts (20–28%, respectively), while only 12% of TTX and trideoxyTTX were detected. In summary, in all *L. sceleratus* specimens, gonads, gastrointestinal tract, and liver were the tissues containing the highest toxin amounts while lower amounts were found in muscle and skin.

Results demonstrated the successful application of the LC–MS method for a reliable quantification of the individual TTXs present in puffer fish.

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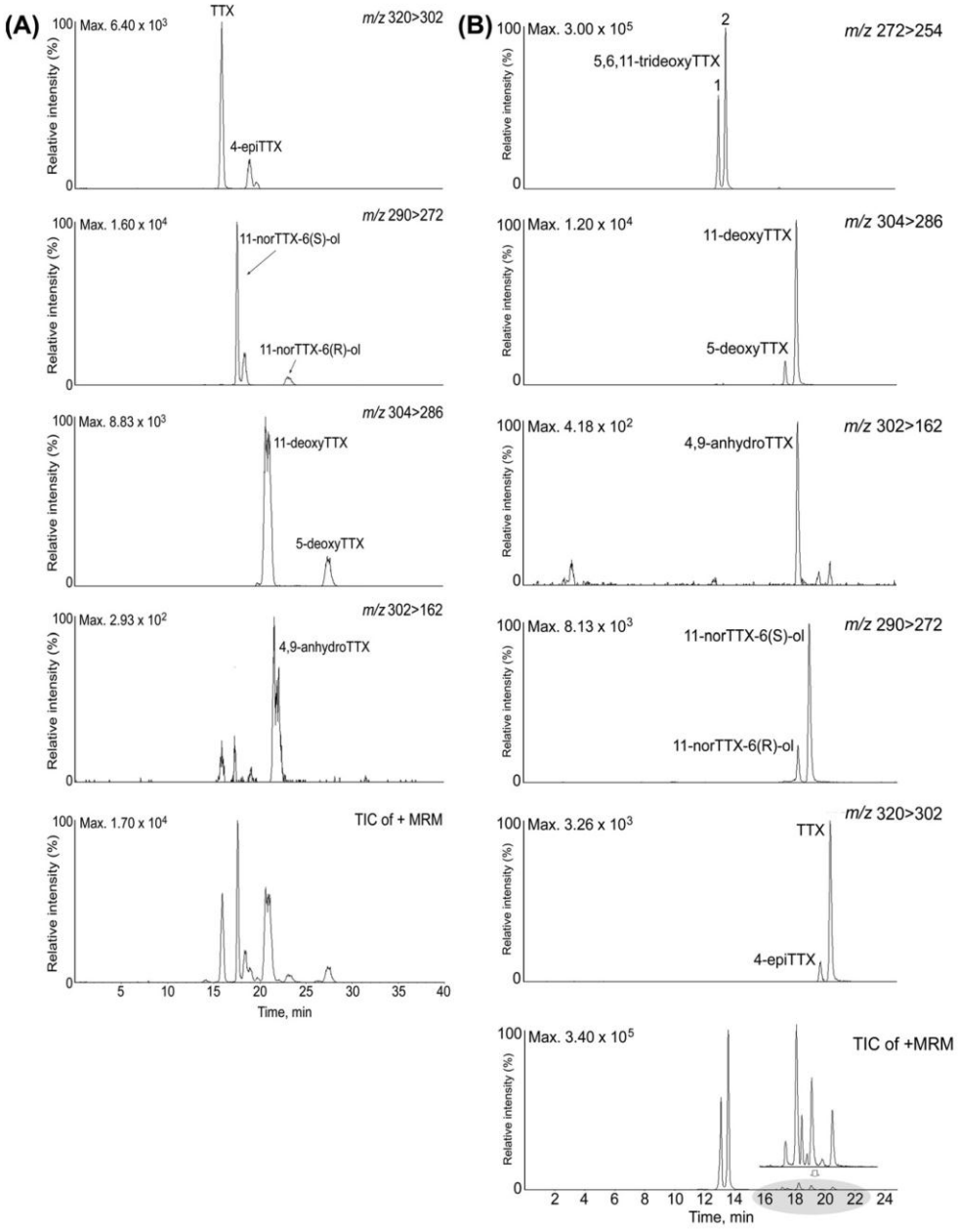

Fig. 5. Mass chromatograms of the LC-ESI-CID-MS/MS obtained under MRM operation of the gonads from No. 6 specimen of *L. sceleratus*. TIC total ion chromatogram. (A) *m*/*z* 290 > 272 for 11-norTTX-6(S)-ol and 11-norTTX-6(R)-ol, *m*/*z* 302 > 162 for 4,9-anhydroTTX, *m*/*z* 304 > 286 for 11-deoxyTTX, and 5-deoxyTTX, *m*/*z* 320 > 302 for TTX, and 4-*epi*TTX; toxins eluted in a Sunfire C18 column (i.d. 4.6 × 250 mm, 5 μm) at 20 °C, 0.4 ml/min. (B) *m*/*z* 272 > 254 for 5,6,11-trideoxyTTX, *m*/*z* 304 > 286 for 5-deoxyTTX, and 11-deoxyTTX, *m*/*z* 302 > 162 for 4,9-anhydroTTX, *m*/*z* 290 > 272 for 11-norTTX-6(R)-ol, and 11-norTTX-6(S)-ol, *m*/*z* 320 > 302 for 4-*epi*TTX, and TTX; toxins eluted in a XBridge Amide column (i.d. 2.1 × 150 mm; 3.5 μm) at 25 °C, 0.2 ml/min. From minute 16 to 22 it is represented a broadening of the chromatogram where the toxins are eluted.

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Table 1
Toxin amount (μg/g) in tissues of *L. sceleratus* specimens (SP) obtained by LC-ESI-CID-MS/MS in MRM mode and by MBA.

SP	Tissues	TTX	4-*epi*TTX	4,9-anhydroTTX	5-deoxy TTX	11-deoxy TTX	11-norTTX-6(R)-ol	11-norTTX-6(S)-ol	5,6,11-trideoxyTTX (1)	5,6,11-trideoxyTTX (2)	MBA* (μg TTXeq per g)
1	Skin	<LOQ	–	–	<LOQ	<LOQ	–	–	<LOD	<LOD	<1.10
	Muscle	<LOQ	–	–	<LOD	<LOQ	–	<LOD	<LOQ	<LOQ	<1.10
	G.I	<LOQ	–	–	–	<LOQ	–	–	<LOD	<LOQ	<1.10
	Liver	0.37	–	–	<LOD	<LOQ	–	0.41	1.65	3.16	<1.10
2	Skin	<LOQ	<LOD	–	<LOQ	0.73	–	0.38	2.43	5.15	<1.10
	Muscle	<LOQ	<LOD	–	<LOD	<LOQ	<LOQ	<LOQ	1.20	2.30	<1.10
	G.I	0.32	<LOD	<LOD	<LOQ	0.43	0.34	0.36	1.70	3.18	<1.10
	Liver	<LOQ	–	–	<LOQ	<LOQ	0.64	0.52	1.29	2.03	<1.10
3	Skin	0.65	<LOD	<LOQ	<LOQ	0.56	1.08	0.83	23.55	56.50	2.34
	Muscle	0.68	<LOD	<LOQ	<LOQ	1.30	–	0.75	19.40	45.60	1.69
	G.I	12.05	2.96	1.97	3.67	17.45	3.53	16.50	109.75	220.75	61.05
	Liver	2.39	0.47	0.52	0.76	2.55	0.36	4.38	94.00	192.25	5.08
4	Skin	0.53	<LOD	<LOD	<LOD	1.55	<LOQ	0.79	15.70	34.70	2.42
	Muscle	0.66	<LOQ	<LOQ	0.57	4.30	<LOQ	0.96	5.00	9.65	2.52
	G.I	19.85	3.20	2.65	7.05	31.80	5.70	37.90	153.75	359.75	56.78
	Liver	7.25	1.47	1.64	6.85	28.55	2.96	16.60	115.25	273.50	16.12
	Gonads	4.12	0.83	0.34	4.20	23.35	1.09	6.95	17.50	66.00	17.05
5	Skin	<LOQ	<LOD	<LOD	–	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<1.10
	Muscle	<LOQ	–	<LOD	<LOQ	0.54	0.47	0.48	<LOQ	<LOQ	<1.10
	G.I	1.09	<LOQ	0.33	0.86	3.18	3.61	3.70	0.37	2.23	6.31
	Liver	4.20	0.46	0.63	1.62	5.50	3.16	8.70	0.71	4.07	10.84
	Gonads	0.47	–	–	0.37	1.11	0.41	0.43	<LOQ	0.33	1.49
6	Skin	1.40	–	<LOD	0.47	3.55	0.45	3.32	8.60	15.55	6.63
	Muscle	3.47	0.41	<LOQ	1.15	8.85	1.41	6.40	8.30	28.45	10.16
	G.I	37.60	5.15	2.28	12.65	72.50	10.75	70.50	86.25	180.75	177.65
	Liver	44.15	7.55	6.60	13.40	92.00	20.10	182.25	412.25	602.50	87.53
	Gonads	46.30	6.15	5.55	20.40	230.88	22.35	221.88	2847.50	4847.50	239.32

–: not detected; LOD: 0.08 μg/g; LOQ: 0.32 μg/g.
* from Ref. (Katikou, et al., 2009)

4. Discussion

The purpose of the present study was to investigate the presence of TTXs in tissues of six specimens of *L. sceleratus* (Gmelin, 1789) captured in European waters by determining the quantities of each toxin using LC–MS.

Results indicated that all puffer fish specimens contained TTX and various TTXs, whereas toxin distribution between either the tissues of the same fish or the six specimens was unequal. Thus, among large fishes, No. 4 and No. 6 accumulated the highest toxin amounts in the gastrointestinal tract and gonads, respectively. This was not the case in fish No. 5, in which the amount of toxins was lower and mostly accumulated in the liver. Specimen No. 3, consisting of a group of small fish, accumulated the highest toxin amounts in the gastrointestinal tract and these toxin levels were even higher than those found in specimen No. 5. Different toxin profiles detected in the *L. sceleratus* specimens could be attributed to the fact that fish were captured in different areas and seasons, since specimen No. 5 was collected in the area of Horeyto, Pelion in June and specimens No. 1–4 and 6 were caught near the island of Rhodes during October–December.

Results obtained by LC–MS were comparable with the previous toxicological analyses of the samples by MBA (Katikou, et al., 2009). The MBA was positive for TTX in all tested tissues except in those from No. 1 and 2 specimens. In this sense, the amount of TTX detected by LC–MS was lower than the LOQ (<0.32 μg/g) in both specimens. Although low amounts of 5,6,11-trideoxyTTX below 5.2 μg/g were detected in the tissues of No. 1 and 2 specimens, this toxin is considered almost a non-toxic analogue (Jang & Yotsu-Yamashita, 2006). For this reason, it is logical that the MBA was negative in these specimens. In all specimens, gonads, gastrointestinal tract, and liver were the tissues where TTXs were found in the highest amount, while muscle and skin contained lower amounts. Internal organs also showed the highest toxicities in the MBA, while toxicity was also present in muscle and skin but was much lower (Katikou, et al., 2009). In other studies with *L. sceleratus* as well as different *Lagocephalus* species, muscle showed some high toxicity levels, although the gonads, liver, and gastrointestinal tract were frequently the most toxic tissues (El-Sayed, Yacout, El-Samra, Ali, & Kotb, 2003; Hwang, et al., 1992; Kanoh, Noguchi, Maruyama, & Hashimoto, 1984; Noguchi, et al., 2006). The analytical results obtained by LC–MS thus reinforce the results of the biological assay since the distribution of the total amount of toxins between the tissues of the six specimens have the same toxic profile to that obtained in the MBA (Katikou, et al., 2009).

For routine analysis of TTX and its analogues many authors have used the LC with fluorescent detection system as the proposed method to replace the conventional mouse bioassay. Nevertheless, the LC–MS is becoming more popular since it offers both sufficient separation and high ionisation intensities of the compounds (Jang, Lee, & Yotsu-Yamashita, 2010; Jang & Yotsu-Yamashita, 2006; Kono, et al., 2008; Shoji, et al., 2001). In this study, sample solutions were first analysed using a previously developed method for detecting TTX and 5,6,11-trideoxyTTX (Rodríguez, et al., 2008). Although TTX was accurately detected, the individual TTXs present in the samples could not be identified, as the toxin separation achieved was not sufficient. The second method employing a Sunfire C18 column and a mobile phase with heptafluorobutyric acid was developed following previous reports (Shoji, et al., 2001). In this case a better separation of the toxins was achieved, though the analogue 5,6,11-trideoxyTTX was retained in the column and could not be accurately identified. Other disadvantages encountered in this method were the low volatility of the mobile phase causing problems in the MS spectrometer, the high variability of the signal obtained in the chromatograms, as well as the long run time of the analysis (40 min).

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The last and new method proposed here, finally, allowed selective and sensitive detection of the TTXs in the MRM mode in 25 min. A good separation of the individual TTXs present in the samples was achieved by use of a Xbridge Amide column, whereas, consistent retention times of the toxins and better resolution of the peaks were also obtained. The composition of the mobile phase, containing low buffer concentration and a high percentage of organic solvent, resulted in high ionisation efficiency and guaranteed low LOD for TTX. With this method, TTX, 4-*epi*TTX, 4,9-anhydroTTX, 5-deoxyTTX, 11-norTTX-6(R)-ol, and 11-norTTX-6(S)-ol were detected in most tested tissues but 5,6,11-trideoxyTTX was found to be the major TTX analogue in all *L. sceleratus* specimens. This toxin was eluted in two different retention times possibly due to the existence of two isomers in equilibrium.

Similar toxin profile was reported in other puffer fish species as the Japanese *Fugu pardalis*, in which the non toxic 5,6,11-trideoxyTTX was the major toxin, while 11-deoxyTTX and 5-deoxyTTX were only detected in the ovaries and liver with 10% and 1% of TTX, respectively (Jang & Yotsu-Yamashita, 2006). These deoxy analogues were also present in the Korean *Fugu niphobles* and in two *Tetraodon* species (*T. nigroviridis* and *T. biocellatus*) but in lower amounts than TTX and 5,6,11-trideoxyTTX (Jang, et al., 2010). In addition, 11-deoxyTTX and 4,9-anhydroTTX were detected in the Blangadesh *Takifugu oblongus*, mostly in the ovary, even though 5,6,11-trideoxy was the toxin dominant in this organ (Diener, Christian, Ahmed, & Luckas, 2007). Data published so far suggest that the deoxy analogues of TTX are commonly present in puffer fishes, although the different ratios between them are specific of the species and/or of the regions where the fish are collected. For this reason, it is possible that the relation of toxins in the puffer fish *L. sceleratus* was different. So, 5,6,11-trideoxyTTX was the main toxin, with two isomers detected followed 11-deoxyTTX and 11-norTTX-6(S)-ol which were found in higher amount than TTX. Further studies would be needed to verify if the toxin profile of the *L. sceleratus* caught in European waters is similar to that obtained here or if instead other TTX analogues are detected in this species.

The trigger of this research was the increased incidence of *L. sceleratus* in Greek waters together with the recent cases of food poisoning by consuming this fish reported from Israeli coast (Bentur, et al., 2008). To our knowledge these are the first studies where the TTX, 4-*epi*TTX, 4,9-anhydroTTX, 5-deoxyTTX, 11-deoxyTTX, 5,6,11-trideoxyTTX (1), 5,6,11-trideoxyTTX (2), 11-norTTX-6(R)-ol, and 11-norTTX-6(S)-ol were detected and quantified in the tissues of the marine puffer fish *L. sceleratus* by LC-ESI-CID-MS/MS in MRM mode.

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References

Akyol, O., Ünal, V., Ceyhan, T., & Bilecenoglu, M. (2005). First confirmed record of *Lagocephalus sceleratus* (Gmelin, 1789) in the Mediterranean Sea. *Journal of Fish Biology, 66*(4), 1183–1186.

Bentur, Y., Ashkar, J., Lurie, Y., Levy, Y., Azzam, Z. S., Litmanovich, L., et al. (2008). Lessepsian migration and tetrodotoxin poisoning due to *Lagocephalus sceleratus* in the eastern Mediterranean. *Toxicon, 52*, 964–968.

Diener, M., Christian, B., Ahmed, M. S., & Luckas, B. (2007). Determination of tetrodotoxin and its analogues in the puffer fish *Takifugu oblongus* from Bangladesh by hydrophilic interaction chromatography and mass-spectrometric detection. *Analytical and Bioanalytical Chemistry, 389*, 1997–2002.

El-Sayed, M., Yacout, G. A., El-Samra, M., Ali, A., & Kotb, S. M. (2003). Toxicity of the Red Sea pufferfish *Pleuranacanthus sceleratus* "El-Karad". *Ecotoxicology and Environmental Safety, 56*(3), 367–372.

Endo, A., Khora, S. S., Murata, M., & Yasumoto, T. (1988). Isolation of 11-nor-tetrodotoxin-6(R)-ol and other tetrodotoxin derivatives from the puffer. *Fugu niphobles Tetrahedron Letter, 29*, 4127–4128.

Galil, B. S., & Zenetos, A. (2002). A sea change: Exotics in the eastern Mediterranean Sea. In E. Leppäkoski, S. Gollasch, & S. Olenin (Eds.), *Invasive Aquatic Species of Europe: Distribution, Impacts and Management* (pp. 325–336). Dordrecht, Netherlands: Kluwer Academic Publishers.

Golani, D., & Levy, Y. (2005). New records and rare occurrences of fish species from the Mediterranean coast of Israel. *Zoology in the Middle East, 36*, 27–32.

Goto, T., Kishi, Y., Takahashi, S., & Hirata, Y. (1965). Tetrodotoxin. *Tetrahedron, 21*, 2059–2088.

Hwang, D. F., Kao, C. Y., Yang, H. C., Jeng, S. S., Noguchi, T., & Hashimoto, K. (1992). Toxicity of puffer in Taiwan. *Nippon Suisan Gakkaishi, 58*, 1541–1547.

Jang, J., & Yotsu-Yamashita, M. (2006). Distribution of tetrodotoxin, saxitoxin, and their analogues among tissues of the puffer fish *Fugu pardalis*. *Toxicon, 48*(8), 980–987.

Jang, J. H., Lee, J. S., & Yotsu-Yamashita, M. (2010). LC/MS analysis of tetrodotoxin and its deoxy analogues in the marine Puffer Fish *Fugu niphobles* from the Southern Coast of Korea, and in the Brackishwater Puffer Fishes *Tetraodon nigroviridis* and *Tetraodon biocellatus* from Southeast Asia. *Marine Drugs, 8*, 1049–1058.

Jang, J. H., & Yotsu-Yamashita, M. (2007). 6,11-Dideoxytetrodotoxin from the puffer fish, *Fugu pardalis*. *Toxicon, 50*, 947–951.

Kanoh, S., Noguchi, T., Maruyama, J., & Hashimoto, K. (1984). Toxicity of a pufferfish, "senninfugu" *Pleuranacanthus sceleratus*. *Nippon Suisan Gakkaishi, 50*, 2139.

Kasapidis, P., Peristeraki, P., Tserpes, G., & Magoulas, A. (2007). First record of the Lessepsian migrant *Lagocephalus sceleratus* (Gmelin 1789) (Osteichthyes: Tetraodontidae) in the Cretan Sea (Aegean, Greece). *Aquatic Invasions, 2*(1), 71–73.

Katikou, P., Georgantelis, D., Sinouris, N., Petsi, A., & Fotaras, T. (2009). First report on toxicity assessment of the Lessepsian migrant pufferfish *Lagocephalus sceleratus* (Gmelin, 1789) from European waters (Aegean Sea, Greece). *Toxicon, 54*(1), 50–55.

Kono, M., Matsui, T., Furukawa, K., Takase, T., Yamamori, K., Kaneda, H., et al. (2008). Examination of transformation among tetrodotoxin and its analogues in the living cultured juvenile puffer fish, kusafugu, *Fugu niphobles* by intramuscular administration. *Toxicon, 52*(6), 714–720.

Laobhripatr, S., Limpakarnjanarat, K., Sangwonloy, O., Sudhasaneya, S., Anuchatvorakul, B., Leelasitor, S., et al. (1990). Food poisoning due to consumption of the freshwater puffer *Tetraodon fangi* in Thailand. *Toxicon, 28*, 1372–1375.

Mahmud, Y., Arakawa, O., & Noguchi, T. (2000). An epidemic survey on freshwater puffer poisoning in Bangladesh. *Journal of Natural Toxins, 9*, 319–326.

Miyazawa, K., & Noguchi, T. (2001). Distribution and origin of tetrodotoxin. *Journal of Toxicology – Toxin Reviews, 20*, 11–33.

Nakamura, M., & Yasumoto, T. (1985). Tetrodotoxin derivatives in puffer fish. *Toxicon, 23*, 271–276.

Noguchi, T., Arakawa, O., & Takatani, T. (2006). TTX accumulation in pufferfish. *Comparative Biochemistry and Physiology, Part D, 1*, 145–152.

Noguchi, T., Jeon, J. K., Arakawa, O., Sugita, H., Deguch, Y., Shida, Y., et al. (1986). Occurrence of tetrodotoxin and anhydrotetrodotoxin in *Vibrio* sp. isolated from the intestine of a xanthid crab, *Atergatis floridus*. *Journal of Biochemistry, 99*, 311–314.

Rodríguez, P., Alfonso, A., Vale, C., Alfonso, C., Vale, P., Tellez, A., et al. (2008). First toxicity report of tetrodotoxin and 5,6,11-trideoxyTTX in the trumpet shell *Charonia lampas lampas* in Europe. *Analytical Chemistry, 80*(14), 5622–5629.

Sabrah, M. M., El-Ganainy, A. A., & Zaky, M. A. (2006). Biology and toxicity of the pufferfish *Lagocephalus sceleratus* (GMELIN, 1789) from the Gulf of Suez. *Egyptian Journal of Aquatic Research, 32*(1), 283–297.

Sherif, N. H. S., Ali, A. E., Abbas, M. M., & Mohamed, A. S. (1994). Studies on the toxins of the puffer fishes in the north western part of the Red Sea. *Journal of Egyptian Germany Society of Zoology, 14*(A), 1–19.

Shoji, Y., Yotsu-Yamashita, M., Miyazawa, T., & Yasumoto, T. (2001). Electrospray ionization mass spectrometry of tetrodotoxin and its analogues: Liquid chromatography/mass spectrometry, tandem mass spectrometry, and liquid chromatography/tandem mass spectrometry. *Analytical Biochemistry, 290*(1), 10–17.

Spanier, E., & Galil, B. S. (1991). Lessepsian migration: A continuous biogeographical process. *Endeavour, 15*, 102–106.

Yasumoto, T., Yasumura, D., Yotsu, M., Michishita, T., Endo, A., & Kotaki, Y. (1986). Bacterial production of tetrodotoxin and anhydrotetrodotoxin. *Agricultural and Biological Chemistry, 50*, 793–795.

Yasumoto, T., Yotsu, M., Murata, M., & Naoki, H. (1988). New tetrodotoxin analogue from the newt *Cynops ensicauda*. *Journal of the American Chemical Society, 110*, 2344–2345.

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Yotsu-Yamashita, M., Schimmele, B., & Yasumoto, T. (1999). Isolation and structural assignment of 5-deoxytetrodotoxin from the puffer fish *Fugu poecilonotus*. *Bioscience, Biotechnology, and Biochemistry, 63*(5), 961–963.

Yotsu-Yamashita, M., Yamagishi, Y., & Yasumoto, T. (1995). 5,6,11-Trideoxytetrodotoxin from the puffer fish, *Fugu poecilonotus*. *Tetrahedron Letters, 36*, 9329–9332.

Yotsu, M., Hayashi, Y., Khora, S., Sato, S., & Yasumoto, T. (1992). Isolation and structural assignment of 11-nortetrodotoxin-6(S)-ol from the puffer *Arothron nigropunctatus*. *Bioscience, Biotechnology, and Biochemistry, 56*, 370–371.

Zaki, A. M. (2004). Tetrodotoxin poisoning associated with eating puffer fish in Suez City (Egypt). In: *1st International Conference on Natural Toxins October 6 University*). Egypt 18–19 December 2004.

3.3. Sección II: Detección y cuantificación de toxinas PSP en diferentes muestras de moluscos y dinoflagelados.

Dentro de las HABs, solo unas pocas especies de dinoflagelados pueden alcanzar altas densidades celulares y sintetizar poderosas toxinas que pueden ser peligrosas para el ser humano. Entre ellas, los dinoflagelados del género *Alexandrium* han sido los más estudiados debido a la amenaza que suponen las toxinas que producen, el largo número de especies y su amplia distribución geográfica [378]. El género comprende aproximadamente 28 especies, pero sólo algunas de ellas son capaces de producir toxinas PSP, otras sin embargo son especies no tóxicas [379]. Aunque a veces se detectan altas densidades celulares de *Alexandrium* en la columna de agua, no siempre se obtienen resultados positivos para toxinas PSP en los MBA realizados en programas de monitoreo. Esto puede ser debido a la convivencia de especies tóxicas con organismos no tóxicos pero de morfología similar. Por otro lado, el perfil de toxinas de los bivalvos y de los dinoflagelados tóxicos es diferente y esto se explica en parte por la transformación química y/o enzimática de las toxinas una vez que estas son retenidas en los tejidos de los moluscos [380, 381]. En general, los moluscos contienen frecuentemente una mayor proporción de toxinas carbamato (o una menor proporción de toxinas N-sulfocarbamoil) en comparación con los dinoflagelados causantes [382, 383].

El creciente número de estudios y el desarrollo de métodos moleculares ha mejorado la capacidad de evaluar las relaciones evolutivas del género *Alexandrium* y la variabilidad de especies [384-388]. En combinación con estos métodos, también se emplean técnicas analíticas para la detección y cuantificación de las toxinas PSP tanto en moluscos bivalvos como en cultivos de dinoflagelados. Habitualmente se utilizan dos métodos de HPLC-FLD para detectar estas toxinas. Por un lado el método de Lawrence, que es el método oficial de la AOAC [287] en el que la oxidación de las muestras se realiza antes de la separación cromatográfica. Y, por otro lado, el método de Oshima [310] en el que la oxidación se produce a la salida de la columna. Ambos métodos presentan numerosas ventajas e inconvenientes.

En este trabajo, se presentan los resultados obtenidos utilizando estas técnicas en el análisis de muestras de moluscos y dinoflagelados.

A esta sección corresponden las siguientes publicaciones:

II.1: Dynamics of co-occurring *Alexandrium minutum* (Global Clade) and *A. tamarense* (West European) (*Dinophyceae*) during a summer bloom in Cork Harbour, Ireland (2006).

II.2: Comparative analysis of pre- and post-column oxidation methods for detection of paralytic shellfish toxins.

II.1: Dinámica de la coexistencia de *Alexandrium minutum* y *A. tamarense* (Europa occidental) (*Dinophyceae*) durante una floración de verano en Cork Harbour, Irlanda (2006).

Resumen

El dinoflagelado del género *Alexandrium* contiene especies productoras de neurotoxinas, que han afectado negativamente a la industria de la acuicultura y a la pesca en todo el mundo. Las floraciones tóxicas, estacionales, de *Alexandrium spp.* se producen anualmente en la zona del Canal Norte del puerto de Cork, Irlanda, donde coexisten poblaciones no tóxicas de *A. tamarense* (ribotipos de Europa Occidental) con *A. minutum* productoras de toxinas PSP. Se realizaron estudios de campo durante una proliferación de *Alexandrium spp.* en el verano del 2006. Para ello, se utilizaron sondas fluorescentes específicas para cada taxón en ensayos de hibridación *in situ* con célula entera para la discriminación y cuantificación simultánea de *A. minutum* y *A. tamarense* en la columna de agua. La floración se produjo después de una marea viva débil a principios de junio y las concentraciones de células de *Alexandrium* excedieron 3 x 10^4 células L^{-1}. *A. minutum* dominó numéricamente sobre *A. tamarense* durante todo el período de muestreo (con un promedio de 74%). La máxima concentración de células fue 3,3 x 10^5 células L^{-1} en el pico de la floración y fue localizada en el extremo oriental del Canal Norte. El colapso de la floración coincidió con el aumento de las mareas y cambios significativos en las condiciones meteorológicas (aumento de la velocidad del viento, menor radiación), lo que llevó a una caída de la temperatura del agua de ~ 3ºC en un periodo de siete días. La GTX3 fue una de las toxinas PSP dominante mientras que las toxinas Cs se observaron ocasionalmente en las

muestras. Suponiendo que *A. minutum* fuera el único microorganismo sintetizador de toxinas PSP, la cuota interna de toxinas estuvo sobre una media de 13,4 fmol cel^{-1}, un valor similar al que se observó en experimentos de laboratorio. De estos resultados se concluye que la monitorización de especies tóxicas de *Alexandrium* en Irlanda requerirá el uso de métodos moleculares para una discriminación y cuantificación fiables.

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Dynamics of co-occurring *Alexandrium minutum* (Global Clade) and *A. tamarense* (West European) (Dinophyceae) during a summer bloom in Cork Harbour, Ireland (2006)

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ABSTRACT

The dinoflagellate genus *Alexandrium* contains neurotoxin-producing species, which have adversely affected the aquaculture industry and fisheries worldwide. Seasonal toxic blooms of *Alexandrium* spp. occur on an annual basis in the North Channel area of Cork Harbour, Ireland, where resident populations of non-toxic *A. tamarense* (West European ribotype) and PSP toxin-producing *A. minutum* (Global Clade) co-occur. Field surveys were carried out throughout a bloom of *Alexandrium* spp. in the summer of 2006. Taxa-specific fluorescently labelled probes were used in a dual whole-cell fluorescent *in situ* hybridization (WC-FISH) assay for the simultaneous discrimination and quantification of *A. minutum* and *A. tamarense* in the water column. The bloom occurred following a weak spring tide in early June and *Alexandrium* cell concentrations exceeded 3×10^4 cells L^{-1}. *A. minutum* dominated numerically over *A. tamarense* throughout the sampling period (74% on average). The maximum cell concentration was $\sim 3.3 \times 10^5$ cells L^{-1} at the peak of the bloom and was localized at the eastern end of the North Channel. The bloom collapse coincided with increasing tidal flushing and significantly changing meteorological conditions (wind speed increase, lesser irradiance), which led to a water temperature drop of $\sim 3\ ^{\circ}C$ within a period of 7 days. GTX3 was the dominant PSP toxin variant and C-toxins were at times observed in samples. Assuming that *A. minutum* was the only microorganism synthesising PSP toxins, the internal toxin quota was on average 13.4 fmol $cell^{-1}$, a value similar to that observed in laboratory experiments. Monitoring of toxic *Alexandrium* species in Ireland will require the use of molecular methods for reliable discrimination and quantification.

1. Introduction

The distribution, intensity and frequency of harmful algal bloom (HAB) events have increased over the past decades worldwide (Anderson et al., 1989; Hallegraeff, 2003). These phenomena have had deleterious effects on flora and fauna in coastal areas and, through mass mortality of stocks or contamination with phycotoxins, have severely affected aquaculture operations in various countries (Shumway, 1990; Hallegraeff et al., 1998; FAO, 2004). The shellfish aquaculture sector is particularly sensitive to toxic algal blooms and as a result phytoplankton and biotoxin monitoring programs have been implemented to protect public health and limit economic losses (Andersen et al., 2003; Smayda, 2003). However, patchiness in the distribution of some toxic species and co-occurrences of morphologically similar organisms constitute difficulties that can hinder the efficiency of monitoring programs. The acquisition of reliable and accurate data through spatial and temporal surveys at sites where risks of HAB occurrences are high is necessary to parameterise predictive bio-physical models of harmful phytoplankton blooms (McGillicuddy et al., 2005).

Paralytic shellfish poisoning (PSP) is a potentially lethal intoxication syndrome that affects humans through consumption of shellfish contaminated with neurotoxins produced by some microalgal species (Wright, 1995; Cembella, 1998). PSP toxins constitute a group of about 20 low molecular weight alkaloid compounds referred to as saxitoxins, which impair the transmission of electric fluxes between muscular and nerve cells by inhibiting the voltage-gated sodium channels in synapses (Luckas et al., 2003). Symptoms in humans include nausea, dizziness, paresthesia, and severe intoxications can lead to death through respiratory failure (Kao, 1993).

The dinoflagellate genus *Alexandrium* comprises ~28 species, some of them with the ability to produce saxitoxins (Balech, 1995). The regulatory limit set for PSP toxins in shellfish tissues is typically 80 μg saxitoxin equivalent per 100 g (STX eq./100 g flesh) (FAO, 2004). Toxic populations of *Alexandrium* spp. have been associated with PSP events on most continents, with shellfish

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contamination levels greater than 3×10^3 μg STX eq. 100 g^{-1} flesh in Scotland, Japan, South Africa or the US east coast having been observed (FAO, 2004). Morphological similarities between *Alexandrium* spp. do not often allow the discrimination between co-occurring species by light microscopy. Therefore, population dynamics studies carried out at the species level are not well documented (see John et al., 2003; Gribble et al., 2005; Anderson et al., 2005a for exceptions).

The increasing use of molecular biology methods in the last decade has contributed to the genetic characterization of several HAB species and the current understanding of relationships between genera, populations and strains (Scholin et al., 1995; Hansen et al., 2000; Edvardsen et al., 2003; John et al., 2004). A number of molecular assays based on the variability of rDNA genes between taxa have enabled the rapid detection and quantification of individual HAB species in complex environmental samples. Assays involving the use of whole-cell fluorescent *in situ* hybridization (WC-FISH), sandwich hybridization (SH), biosensors or real-time PCR have been developed for *Alexandrium* spp. and successfully applied to the analysis of natural populations (Anderson et al., 2005b; John et al., 2005; Dyhrman et al., 2006; Lazerges et al., 2006; Diercks et al., 2008). WC-FISH, which allows the specific detection of target taxa through the binding of oligonucleotide probes to the rRNA component of ribosomes present in actively growing cells, is a popular method for ecological studies (Sanz and Köchling, 2007; Wakeham et al., 2007). Assay design and protocol optimisation are less tedious compared to more sophisticated methods and involve relatively low costs. The non-disruption of cells in samples also offers the possibility of subsequent analysis by additional techniques.

In Ireland, PSP contamination of shellfish occurs on an annual basis in Cork Harbour, an inlet located on the south coast of the country, and seems to be caused by a population of *A. minutum* (Global Clade, Lilly et al., 2005), which co-occurs with a non-toxic population of the West European ribotype (W.E.) of *A. tamarense* there (Touzet, 2006). This clade has recently been termed "Group 2" by Lilly et al. (2007) because geographically based names can be misleading. Studies have shown that blooms originate from the North Channel area of the harbour, where high-density *Alexandrium* spp. resting cyst beds can be found (Ní Rathaille, 2007). However, the population dynamics of *A. minutum* and *A. tamarense* through bloom events are as yet unknown. This study documents the respective distribution and abundance of both species during a bloom in summer 2006 in Cork Harbour. Some environmental parameters (temperature, salinity, chlorophyll fluorescence) were monitored *in situ* in conjunction with the laboratory-based determination of PSP toxin, nutrient and *Alexandrium* spp. concentrations.

2. Methods

2.1. Study area

Samples were collected near low tide in the North Channel area of Cork Harbour, Ireland, during seven surveys at approximately weekly intervals from 25 May to 7 July 2006 (Table 1, Fig. 1). Cork Harbour is an industrialised (oil refinery, fret activities) natural harbour of ~100 km^2 surface, which opens southward to the Celtic Sea. It is partitioned into three connected water basins: the main harbour, Lough Mahon and the North Channel. The main freshwater source is the Lee River, which flows from Cork City into Lough Mahon. The average tidal range for Cork Harbour is 3.7 and 2.0 m on spring and neap tides, respectively. It is a shallow harbour with hydro-dynamically scoured channels; the main channel is 10–20 m deep and the rest of the harbour has a depth comprised between 0 and 5 m at low water. Intertidal mudflats are exposed at low tide and cover a ~40% area of the North Channel. Cork Harbour is the only area in the Republic of Ireland where a ban on shellfish harvesting has been enforced due to the presence of PSP toxins.

Table 1
Details of the stations sampled during the surveys carried out in Cork Harbour in summer 2006.

Date (survey)	Station	Location	Time (local)	Latitude (N)	Longitude (W)
25 May 2006 (01)	4201	O	10:07	51°52.825′	8°15.046′
	4202	OP	10:36	51°52.883′	8°14.492′
	4203	P	10:58	51°52.922′	8°13.957′
	4204	PQ	11:19	51°52.911′	8°13.254′
	4205	Q	11:41	51°52.895′	8°12.891′
	4206	QR	12:06	51°52.941′	8°12.477′
	4207	R	12:26	51°52.928′	8°12.06′
	4208	T	13:11	51°52.169′	8°12.367′
31 May 2006 (02)	4307	T	13:56	51°52.190′	8°12.341′
	4308	R	14:23	51°52.914′	8°12.088′
	4309	Q	14:52	51°52.888′	8°13.004′
	4310	PQ	15:15	51°52.852′	8°13.508′
	4311	P	15:40	51°52.908′	8°14.031′
	4312	OP	16:00	51°52.906′	8°14.554′
	4313	O	16:20	51°52.817′	8°15.029′
8 June 2006 (03)	4403	OP	09:28	51°52.894′	8°14.585′
	4404	P	09:49	51°52.908′	8°14.030′
	4405	PQ	10:23	51°52.922′	8°13.501′
	4406	Q	10:57	51°52.928′	8°12.933′
	4407	R	11:22	51°52.922′	8°12.064′
	4408	T	11:57	51°52.168′	8°12.362′
15 June 2006 (04)	4508	T	13:32	51°52.176′	8°12.353′
	4509	R	14:07	51°52.917′	8°12.088′
	4510	Q	14:42	51°52.915′	8°12.939′
	4511	PQ	15:09	51°52.920′	8°13.474′
	4512	P	15:40	51°52.910′	8°13.970′
	4514	O	16:14	51°52.824′	8°15.026′
22 June 2006 (05)	4601	OP	08:41	51°52.933′	8°14.489′
	4602	P	09:04	51°52.910′	8°13.986′
	4603	PQ	09:34	51°52.938′	8°13.507′
	4604	Q	10:09	51°52.904′	8°13.011′
	4605	R	10:48	51°52.927′	8°12.071′
	4606	T	11:28	51°52.182′	8°12.320′
29 June 2006 (06)	4708	T	13:25	51°52.195′	8°12.344′
	4709	R	13:57	51°52.940′	8°12.092′
	4710	Q	14:41	51°52.897′	8°12.998′
	4711	PQ	15:07	51°52.925′	8°13.542′
	4712	OP	15:35	51°52.868′	8°14.428′
7 July 2006 (07)	4801	LAGOON[a]	10:00	51°53.050′	8°14.500′

[a] Sampling was carried out periodically at a fixed position from low to high tide; temperature, salinity and *Alexandrium* spp. concentrations only were measured.

2.2. Meteorological data and water column sampling

Daily values of incident irradiance data were obtained using a Licor LI-190 Quantum Sensor, which was located midway on the north shore of the North Channel. Wind speed data were obtained from Cork airport, west of Cork Harbour.

Temperature and salinity were measured *in situ* by means of a calibrated probe (WTW, Cond197i). The vertical profiling of chlorophyll fluorescence was carried out using a fluorometer (FL500 SeaTech, Oregon). Samples for chlorophyll-*a* analysis were obtained by filtering 500 mL seawater through GF/C filters. Filters were temporarily kept in a refrigerated box and stored at −32 °C in the laboratory until analysis. Seawater samples (50 mL) were passed through 0.45-μm syringe filters, kept in a refrigerated box and also stored at −32 °C for inorganic nutrient analysis (nitrate,

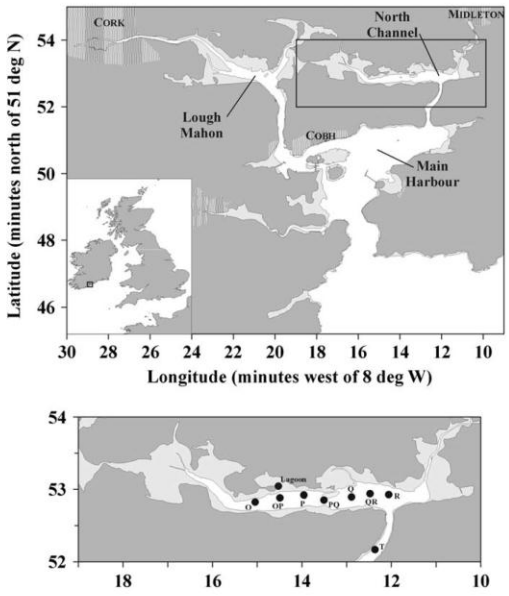

Fig. 1. Map of the study area indicating the stations sampled in the summer 2006. The solid light grey zones highlight the areas exposed at low tide. The bottom panel shows an enlarged view of the North Channel area of Cork Harbour.

nitrite, phosphate). For ammonia concentration determination, seawater was filtered through 0.45-μm syringe filters into 30-mL glass universal containers into which phenol and alkaline hypochlorite reagents (1 mL) were sequentially added and immediately homogenised (Riley, 1953). Samples were kept in the dark in a refrigerated box for at least 24 h before spectrophotometric measurement of the indophenol blue colour. Surface seawater samples (50 mL) were fixed with buffered formalin (1% final concentration $^{v}/_{v}$) for microscopic analysis of the *Alexandrium* spp. community. Samples for the detection and enumeration of *Alexandrium* by WC-FISH were prepared by passing 2 L of seawater through a 150-μm mesh sieve and material was collected onto a 5-μm mesh filter (47 mm diameter). The retained planktonic material was backwashed into 50-mL polypropylene tubes with 0.22 μm filtered seawater and fixed with formalin (0.5% final concentration $^{v}/_{v}$). Samples were kept in the dark until further processing on land. The same procedure, without the formalin fixation step, was used to obtain surface samples for PSP toxin analysis. Samples were stored subsequently at −32 °C in the laboratory.

2.3. Morphotaxonomy-based quantification of Alexandrium spp.

Alexandrium identification and quantification was performed using an Utermöhl sedimentation chamber (Utermöhl, 1958). Armoured dinoflagellates were stained with calcofluor white (Fritz and Triemer, 1985) and *Alexandrium* spp. cells were examined and enumerated using an inverted microscope (Olympus CKX-41) fitted with epi-fluorescence optics. Identification at the species level was based on Balech's (1995) description of the genus and performed by rotating each *Alexandrium* cell with a dissecting needle to observe the morphological characteristics of the thecal plates. Species were differentiated by the presence or absence of a ventral pore on the first apical plate (1′) and the shapes of the sixth precingular (6″) and posterior sulcal plates (sp) (Kofoid notation). Observations were made at 200× and 400× magnifications.

2.4. Whole-cell FISH analysis and epi-fluorescence microscopy

Once back on land, samples for WC-FISH analysis were centrifuged (4000g, 5 min) and the supernatants discarded by aspiration. Pellets were treated with 15 mL 100% ice-cold methanol to extract pigments and stabilise nucleic acids, then stored at −32 °C. The taxa-specific probes MinA (5′-TTATATGGTT-GATGTGGGTGC-3′) and TamA (5′-TAGGTTTTGGCTGTGGGTGA-3′), labelled at the 5′ and both 5′ and 3′ ends with CY.3 and FITC, respectively, were used to carry out hybridizations according to a modified version of the protocol described in Miller and Scholin (1998). Aliquots were placed onto 13-mm-diameter polycarbonate membranes (1.2 μm pore size) held in a custom-made vacuum manifold, and gentle vacuum was applied to remove liquid. Membranes were incubated at room temperature for 1 min with 400 μL hybridization buffer (5X SET, 0.1% IGEPAL, 25 μg mL^{-1} polyadenylic acid) and filtered through. Hybridization buffer containing 200 ng of each probe was then added to each filter prior to incubation into a dark chamber (55 °C, 60 min). After hybridization, the liquid was filtered through and the membranes washed for 1 min with 400 μL 0.2X SET at 55 °C to eliminate excess unbound probes. Filters were removed from the manifold and placed onto slides. Before mounting the coverslip, 5 μL of a mix of calcofluor (100 μg mL^{-1}) and 4′,6-diamidino-2-phenylindole (DAPI, 3 μg mL^{-1}) were added onto each filter as well as 10 μL of SlowFade® Light Antifade (Molecular Probes) reagent to prevent the fading of the dyes' fluorescence.

Membranes were examined with a microscope (Olympus CKX-41) fitted with an epi-fluorescence attachment (U-RFL-T), a 100 W mercury lamp and three-way slider filter holder. The following filter combinations were used for the detection of the fluorescent signals: calcofluor and DAPI (355DF25 excitation filter, 400DRLP dichroic mirror and 420 band pass barrier filter), FITC (485DF22 excitation filter, 505DRLP dichroic mirror and 530DF30 barrier filter) and CY.3 (525AF45 excitation filter, 560DRLP dichroic mirror and 595AF60 barrier filter). Observations were performed at 200× magnification by scanning the entire filter and counting all positive signals. Probe specificity was confirmed with calcofluor by a quick inspection of the general organization of the *Alexandrium* plate tabulation for each positive signal recorded.

2.5. PSP toxin analysis

Samples for PSP toxin analysis were centrifuged (4000g, 5 min) and the supernatants discarded. Pellets were re-suspended with 500 μL of 0.05 M acetic acid to extract toxins and kept at −32 °C until analysis. PSP toxin analysis was carried out by high-performance liquid chromatography (HPLC) with post-column derivatisation using a 5 μm AquaSep column (250 × 4.6 mm i.d., ES Industries Chromega Columns) and fluorometric detection (FD). Analytical details were based on those of Oshima (1995) to identify PSP toxins. Prior to chromatographic separation, impurities in samples were removed by filtration (0.45 μm). The mobile phase for identifying the gonyautoxins group (GTX) was constituted of 2 mM n-heptanesulphonic acid and 10 mM o-phosphoric acid (pH 7.2 with 1 N NH_4OH). The mobile phase used to identify the saxitoxin group (STX) was made of 2 mM n-heptanesulphonic acid, 10 mM o-phosphoric acid and 10% acetonitrile (pH 7.2 with 1 N NH_4OH). The injection volume was 10 μL. The flow rate was adjusted at 0.8 mL min^{-1}. A solution of 7 mM

periodic acid and 50 mM sodium phosphate (pH 9.0 with 1 N NaOH) was introduced to the column eluate in a T made of teflon at a flow rate of 0.8 mL min^{-1}. The resulting mixture was heated while passing through the teflon tubing (10 m × 0.5 mm i.d.) immersed in a water bath at 70 °C. The reaction mixture was then acidified in another T with 0.5 N acetic acid (flow rate 0.1 mL min^{-1}) to reach a pH outflow of 5–7. The eluted fluorescent derivatives were monitored using a Shimadzu RF535 fluorescence detector at 340 and 410 nm excitation–emission wavelengths, respectively. Standards of GTX1 and 4, GTX2 and 3, GTX5 and dc-GTX2 and 3, STX, neo-STX and dc-STX from the Institute for Marine Biosciences, Halifax, Canada, were used to identify each toxin.

2.6. Chlorophyll-a and nutrient analysis

Chlorophyll-*a* concentrations were determined after overnight extraction of the GF/C filtered samples with 90% acetone. Analysis was carried out with a Hitachi U-1100 spectrophotometer at 665 and 750 nm (Strickland and Parsons, 1972). Dissolved inorganic nitrite/nitrate and phosphate were measured by flow injection using a LaChat QC 8000 automated analyser with computer-controlled sample selection and peak processing with the methods recommended by the manufacturer. Ammonia was measured by spectrophotometry at 630 nm (Riley, 1953).

2.7. Statistical analysis

The statistical analyses were performed using SPPS Version 15.0 for Windows. Independent sample Student's *t*-tests were used to compare the average wind speed and global radiation before and after the peak of the *Alexandrium* spp. bloom (3–17 and 18–24 June periods, respectively). One-way analyses of variance were performed to identify significant differences between (1) the average *Alexandrium* spp. concentrations in the North Channel between surveys and (2) the average PSP toxin concentrations and internal quotas in *A. minutum* on different sampling occasions. The data were tested for homogeneity of variance prior to performing post-hoc tests. Square root transformation of the *Alexandrium* spp. concentration data and Tamhane post-hoc test for sample sets of unequal variance were applied.

3. Results

3.1. Evolution of the physico-chemical parameters in the North Channel

Salinity and temperature values fluctuated during the sampling period between 27.4 and 32.1 and between 11.9 and 19.0 °C, respectively (Fig. 2). Salinity progressively increased over time while temperature rose gradually towards 18.0 °C between 26 May and 8 June, dropped down to 15.2 °C on 22 June, and then increased to reach its maximum on 7 July. Average dissolved inorganic nitrogen (DIN) and phosphate concentrations in the North Channel followed an inverse pattern to that of chlorophyll-*a* concentrations (Fig. 2). Both dropped between 31 May and 15 June from 66.9 and 0.74 μM to 8.7 and 0.08 μM, respectively. They then reached 25.7 and 0.55 μM, respectively, during the collapse of the chlorophyll-*a* concentration peak, and decreased again between 22 and 29 June. A positive relationship was observed between the chlorophyll-*a* concentrations derived in the laboratory and the chlorophyll fluorescence recorded *in situ* (r^2=0.916, $p<0.01$).

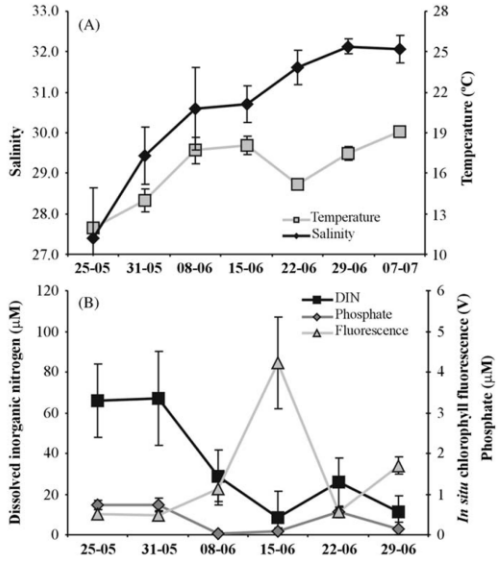

Fig. 2. Evolution of the hydrographic data relative to the North Channel. (A) Average temperature and salinity. (B) Average concentrations of dissolved inorganic nitrogen, phosphate and *in situ* chlorophyll fluorescence.

3.2. Alexandrium spp. bloom development in the North Channel

Intense fluorescent signals were obtained after the hybridization of probes MinA and TamA to *A. minutum* and *A. tamarense* vegetative cells present in the North Channel field samples (Fig. 3). Cells were clearly recognizable and their enumeration straightforward at 200× magnification under epi-fluorescence microscopy. No apparent cross-reactivity with other phytoplankton species was observed as only weak green or orange autofluorescence was detected for non-target taxa. The simultaneous use of DAPI and calcofluor also permitted the visual confirmation of species identification and the reliable discrimination between autofluorescent detritus and target cells.

The average *Alexandrium* sp. concentration derived from the WC-FISH analysis was greater than 5×10^3 cells L^{-1} between 8 and 29 June, with the peak of the bloom most likely occurring between 15 and 22 June (Fig. 4). The evolution patterns of the *Alexandrium* sp. and chlorophyll-*a* concentrations did not match well, the highest average *Alexandrium* sp. density (~90×10^3 cells L^{-1}) occurring at the time when the chlorophyll-*a* concentration was near its minimum. *A. minutum* largely dominated the *Alexandrium* sp. community in the North Channel over the sampling period, with an average contribution of 74% (s.d. 16, n=77). Cell concentrations derived with the WC-FISH method were compared to those obtained using calcofluor and a standard sedimentation chamber. A positive relationship was observed with the FISH-derived results underestimating the cell concentration by a factor of ~1.45. Due to the heterogeneous distribution of *Alexandrium* spp. in the North Channel, the analysis of variance showed that the average cell concentration at the time of the peak of the bloom was not significantly different from those obtained on 15 and 29 June surveys (ANOVA, F=2.80, $p>0.05$) (Table 2). The bloom started to decline following a significant

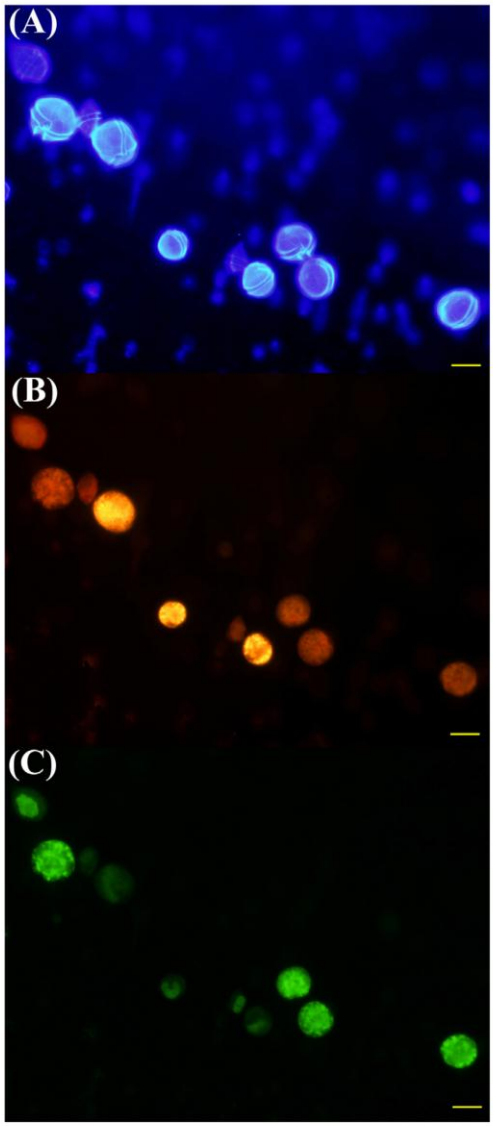

Fig. 3. Epi-fluorescence images of a field sample from Cork Harbour containing *A. tamarense* and *A. minutum* vegetative cells labelled with the probes MinA (orange) and TamA (green). Visualization and discrimination of cells with calcofluor (A), CY.3 (B) and FITC (C) filter sets. Scale bars represent 20 μm.

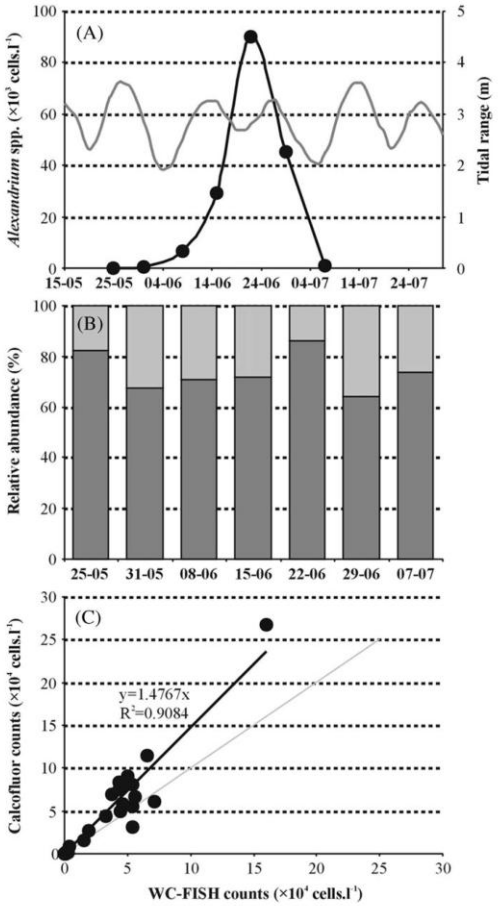

Fig. 4. *Alexandrium* spp. in the North Channel of Cork Harbour during summer 2006. (A) Evolution of average *Alexandrium* spp. cell concentrations derived by WC-FISH (●) and tidal range (–). (B) Relative abundances of *A. minutum* (■) and *A. tamarense* (□). (C) Relationship between the WC-FISH-derived *Alexandrium* spp. concentrations and those obtained using the Utermöhl and calcofluor method.

change in irradiance levels and wind speed near 18 June (*t*-test, $p < 0.05$ and $p < 0.01$, respectively), which led to the decrease of the water temperature by ~3 °C between 15 and 22 June (Table 3, Fig. 2).

3.3. Variable distributions at the peak of the bloom

Vertical profiles performed on 22 June along the transect of stations showed thermal homogeneity in the North Channel (range of 14.9–15.2 °C) as opposed to the previous weeks when the surface temperature was higher and the water column more stratified (data not shown). Higher DIN concentrations were associated with lower surface salinity (29.80) at the eastern end of the channel (station 4605, > 40 μM). *In situ* chlorophyll fluorescence measurements there showed an increased algal biomass, which coincided with the maximum *Alexandrium* spp. cell concentration (~3.3×10^5 cells L^{-1} at 1 m depth). Phosphate concentrations reached 0.70 μM at the western stations sampled in the North Channel and were ~0.30 μM at the eastern side

Table 2
Average concentrations and concentration ranges of *Alexandrium* spp. in the North Channel during summer 2006.

Survey	Average concentration (cells l^{-1})		Concentration range (cells l^{-1})	
	A. minutum (s.d., *n*)	*A. tamarense* (s.d., *n*)	*A. minutum*	*A. tamarense*
25 May 2006	155 (121, 11)	41 (50, 11)	15–377	0–145
31 May 2006	212 (144, 11)	107 (67, 11)	30–459	0–207
8 June 2006	4945 (5288, 11)	1513 (1774, 11)	326–14,438	69–4713
15 June 2006	21,505 (12,907, 12)	7657 (3889, 12)	3149–43,884	1005–15,930
22 June 2006	80,615 (83,540, 11)	9445 (3776, 11)	31,831–142,656	5400–18,090
29 June 2006	29,556 (10,132, 8)	15,749 (4061, 8)	14,575–41,736	9035–20,838
7 July 2006	607 (375, 13)	221 (155, 13)	175–1415	66–548

Table 3
Average wind speed at Cork airport and daily global radiation recorded near the time of the summer 2006 *Alexandrium* spp. bloom in the North Channel.

Period	Wind speed[a] in Knots (s.e., *n*)	Daily global radiation in J cm^{-} (s.e., *n*)
3–17 June	4.1 (0.18, 360)	2169 (179, 15)
18–24 June	7.2 (0.34, 168)	1372 (187, 7)
Student *t*-test	**	*

* and ** indicate significance for $p < 0.05$ and $p < 0.01$, respectively.

[a] East–west axial wind component (North Channel orientation).

towards the main harbour. The phosphate concentration was 0.56 μM in the vicinity of the river plume where the dense patch of *Alexandrium* spp. was localized. The FISH analysis showed the dominance of *A. minutum* which was maximal at the east end of the north channel near the river plume (~95%), the contribution of *A. tamarense* being on average 14% (s.d. 6, $n=11$) (Fig. 5).

3.4. Spatial distributions of A. minutum and A. tamarense

Fig. 6 shows the abundances and vertical distributions of *A. minutum* and *A. tamarense* along each transect of stations carried out during three surveys. On 8 June, the maximum densities of *A. minutum* and *A. tamarense* did not coincide spatially. *A. tamarense* was mostly present at 2 m depth at both ends of the North Channel whereas *A. minutum* seemed to be localized midway into two surface and sub-surface patches. On 15 June, the maximum concentrations of both species were found at the shallow western stations of the North Channel. At the peak of the bloom on 22 June, the maximum density of *A. minutum* was localized at the eastern end of the North Channel while the distribution of *A. tamarense* was more homogeneous.

3.5. Analysis of PSP toxins

PSP toxins were detected in all the surface samples collected in the North Channel during the sampling period. Reliable quantification of the dominant toxin variants GTX2–3 was only possible for the samples collected during surveys 4, 5 and 6 due to low *Alexandrium* concentrations on other sampling occasions. No significant difference was found in the average concentration of PSP toxins in the North Channel for these three dates (ANOVA, $F=2.4$, $p>0.05$) (Table 4). Assuming that only *A. minutum* produced PSP toxins, the estimated average cellular toxin quota derived from the cell concentrations obtained using the WC-FISH method was 13.4 fmol $cell^{-1}$ (10.8 s.d., $n=14$). However, the average PSP toxin quota derived from samples collected on 15 June was significantly higher than those obtained for 22 and 29 June (ANOVA, $F=16.3$, $p<0.001$). Only a very weak positive relationship ($r^2=0.28$, $n=14$, $p<0.05$) was found between PSP toxin levels and *A. minutum* concentrations while considering data from the three surveys (Fig. 7). Excluding data from 15 June, the relationship was better supported ($r^2=0.87$, $n=9$, $p<0.01$) but biased by the influence of the high cell concentration from the sample collected at station 4605. GTX3 was dominant in samples (average 70%, s.d. 12, $n=14$) and traces of C-toxins were also detected in some samples (Fig. 8). Taking the data from surveys 4–6 together, a weak positive relationship was visible between the estimated *A. minutum* PSP toxin quotas and *in situ* surface N:P ratios ($r^2=0.40$, $n=14$, $p<0.01$). Taken individually for the same dates, a significant negative correlation was observed between *A. minutum* PSP toxin quotas and phosphate concentrations ($r^2=0.34$, $n=14$, $p<0.05$), whereas no apparent relationship was visible with DIN ($r^2=0.002$, $n=14$, $p>0.05$).

4. Discussion

4.1. Molecular-based quantification and count calibration

The identification of *Alexandrium* spp. in seawater samples by conventional light microscopy is complex and time consuming, particularly when co-occurring species are found with other morphologically similar dinoflagellates such as *Scrippsiella* sp., *Protoperidinium* sp. or *Gonyaulax* sp. (Anderson et al., 2005b). These difficulties are also compounded by the fact that *Alexandrium* spp. often account for a minor proportion of natural phytoplankton assemblages. Molecular methods relying on sequence heterogeneity within the ribosomal genes and RNA subunits have proved the most popular for HAB species detection and quantification due to the existence of both conservative and variable regions in target sequences (Scholin and Anderson, 1996; Edvardsen et al., 2003; McDonald et al., 2007). Several qRT-PCR assays available for a few *Alexandrium* spp. offer high sample throughput and seem ideal for taxa-specific monitoring and analysis of samples generated during extensive field surveys (Galluzzi et al., 2004; Hosoi-Tanabe and Sako, 2005; Dyhrman et al., 2006; Erdner et al., 2010). However, calibration of results against data obtained using conventional cell counting methods has shown both under- and over-estimations of concentrations of target taxa, probably reflecting PCR inhibition by humic substances, variability in nucleic acid extraction or variation in the frequency of diploid sexual stages in populations (Moorthi et al., 2006). The specificity of the probes MinA and TamA for the discrimination and quantification of *A. minutum* (GC) and *A. tamarense* (WE) has been reported in a previous study (Touzet et al., 2008). As opposed to qPCR methods which measure released double-stranded nucleic acids in samples, WC-FISH permits the enumeration of live cells with ribosomal activity. Although not as rapid as automated molecular methods such as qPCR and sandwich hybridization assays, WC-FISH offers specific advantages for small-scale field surveys. Morphometry data on

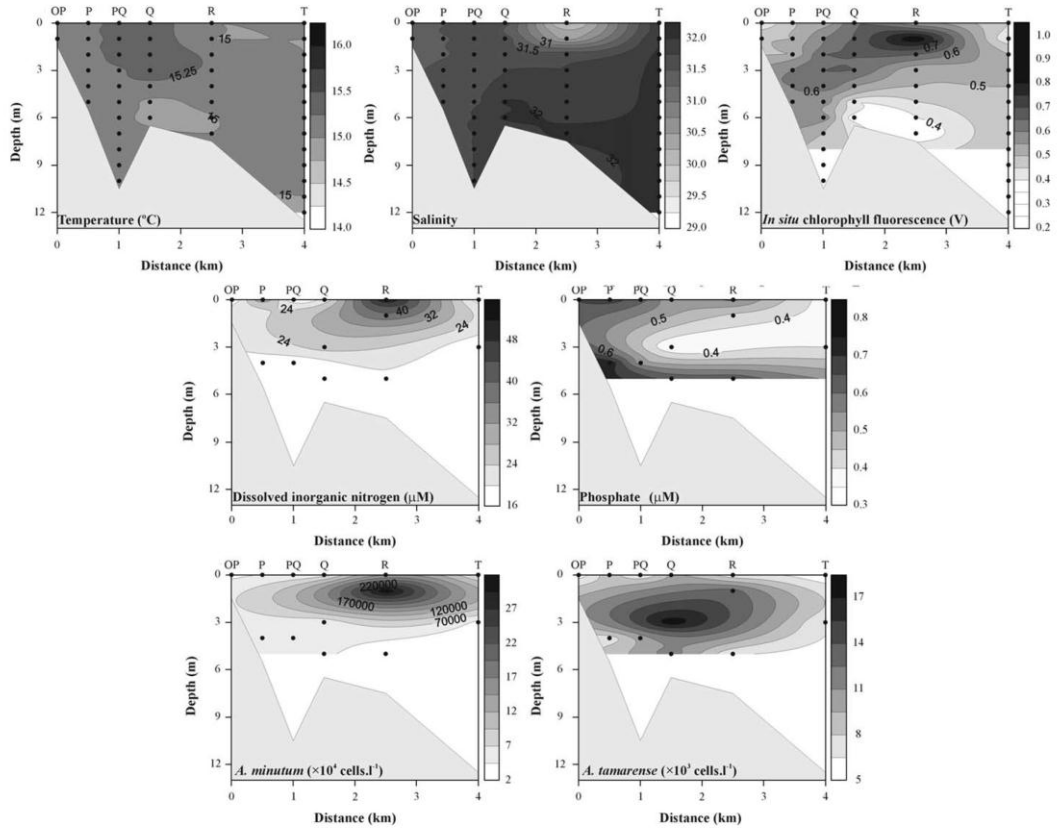

Fig. 5. Vertical distributions of selected environmental variables and WC-FISH-derived *Alexandrium* spp. concentrations along a transect of stations carried out in the North Channel of Cork Harbour on 22 July 2006.

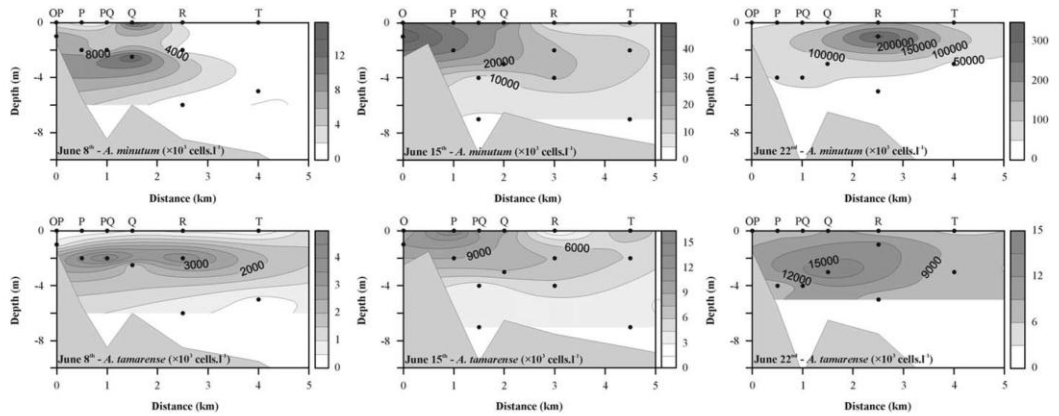

Fig. 6. Distributions of *Alexandrium* spp. along transects of stations carried out in the North Channel of Cork Harbour during the bloom development phase in June 2006.

Table 4
Average PSP toxin concentrations, *A. minutum* internal quotas and GTX3 relative abundances in the North Channel area of Cork Harbour.

Survey	PSP toxin concentration pmol l^{-1} (s.d., *n*)	PSP toxin quota fmol $cell^{-1}$ (s.d., *n*)	GTX3 relative abundance % (s.d., *n*)
15 June 2006	522.6 (235.0, 5)	25.4 (9.2, 5)	74 (19, 5)
22 June 2006	415.2 (266.0, 5)	6.3 (1.1, 5)	64 (4, 5)
29 June 2006	205.7 (81.0, 4)	7.2 (3.4, 4)	71 (8, 4)

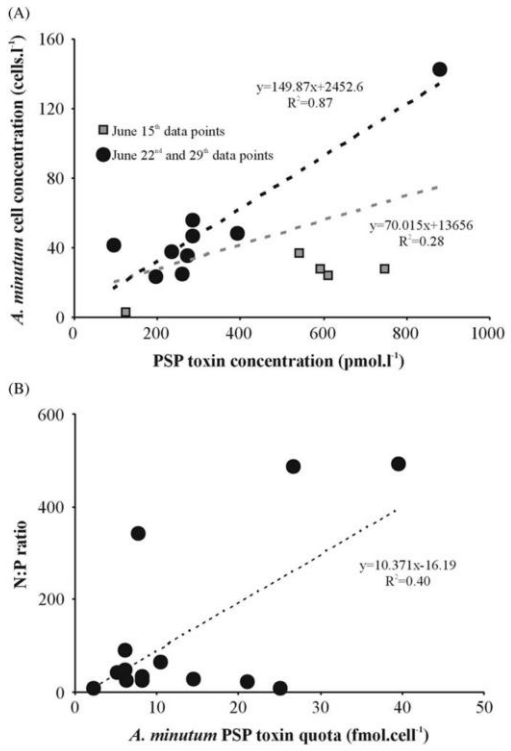

Fig. 7. Relationships between FISH-derived *Alexandrium* spp. cell abundances and PSP toxin concentrations including/excluding 15 June data points (A), and between *A. minutum* cellular PSP toxin quotas and *in situ* N:P ratios (B).

target taxa can easily be gathered to assess the contributions of different life cycle stages to a developing population. The simultaneous use of the cellulose-staining dye calcofluor also offers the possibility of identifying and enumerating the associated armoured dinoflagellate community for comparative dynamics studies.

Few studies have reported the use of WC-FISH for the quantification of *Alexandrium* spp. in water column samples (John et al., 2003; Anderson et al., 2005b; Gribble et al., 2005). Calibration against a reference method is necessary when developing new assays. Previous investigations have usually reported significant underestimations of *Alexandrium* spp. concentrations when using WC-FISH (John et al., 2003; Anderson et al., 2005b). This may be due to cell losses during sample processing or to the non-detection of cells with low ribosomal activity. In this study the overall underestimation factor of *Alexandrium* spp. obtained by WC-FISH against morphotaxonomy analysis using an Utermöhl chamber and calcofluor was near 1.45. This is an improvement compared to the ~2 factor obtained in 2005 during previous surveys when a 10 μm mesh filter of greater surface was used (Touzet et al., 2008). Given the small size of *A. minutum* cells (13–29 μm range dimensions), the use of 5 μm mesh filters proved more efficient for maximizing cell recovery.

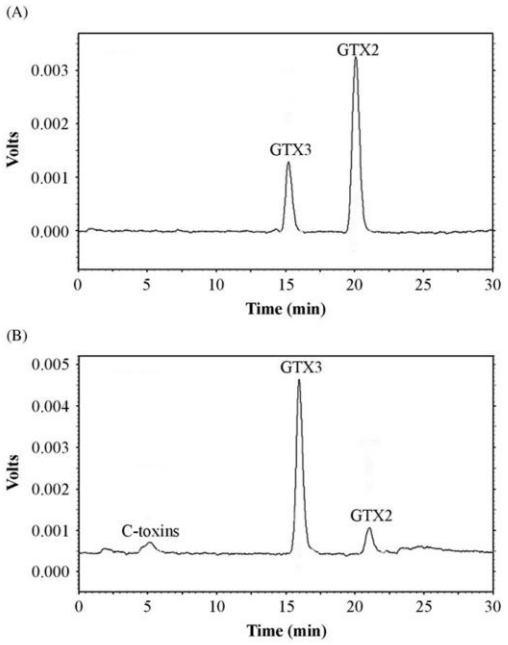

Fig. 8. Selected chromatograms from the HPLC-FD analysis of PSP toxins in field sample extracts. (A) Standards of toxins. (B) Sample from station 4510.

4.2. Bloom dynamics and influence of environmental pressures

Temperatures greater than 15 °C and relatively weak tidal flushing in the North Channel were associated with the *Alexandrium* spp. bloom during summer 2006. Previous studies have shown that blooms mainly develop in the North Channel in the period of June–July, often reaching cell concentrations greater than 1×10^5 cells L^{-1} (Ní Rathaille, 2007). It has also been proposed that the balance between growth rates, as a function of temperature and irradiance, and tidal dilution, exerts the most control over the population development of *Alexandrium* spp. in the area (Ní Rathaille et al., 2008). It has been suggested that the cell inoculum necessary for bloom initiation and development could be provided by the germination of cysts in surface sediments (Anderson, 1998). Large *A. minutum* cells (diameter near 35 μm) labelled with a high orange fluorescence intensity were often visible in the WC-FISH preparations of samples collected in late May. It is possible that those cells may have been recently hatched planomeiocytes with high ribosomal

activity given that the samples collected in June singularly lacked the presence of such large and bright *A. minutum* cells.

The observed maximum *Alexandrium* sp. concentration in the North Channel on 22 June was limited to 3.3×10^5 cells L^{-1}, a concentration below that typically found in other European countries such as Spain and France, where cell densities greater than 1×10^6 cells L^{-1} are often recorded (Probert, 1999; Vila et al., 2005). Numerically, *A. minutum* dominated the *Alexandrium* community. Assuming that an average *A. tamarense* cell is twice as large as an *A. minutum* one, the relative biovolume of both species may be more similar (Touzet, 2006). Supplies of the macronutrient nitrate and phosphate were limiting for phytoplankton growth on several occasions in the North Channel during the sampling period. Redfield ratios were on average well in excess of 16 during each survey, indicating a degree of phosphorus limitation (data not shown). An inverse relationship between nutrient and chlorophyll-*a* concentrations, indicative of nutrient drawdown, was observed; yet none was apparent for *Alexandrium* spp. The low phosphorus concentrations encountered on 8 and 15 June did not prevent the *Alexandrium* bloom, which usually occurs after the first spring tide in early/mid June when tidal flushing is less inhibitory to population growth (Ní Rathaille, 2007). This suggests, at least for estuarine areas, that macronutrient concentrations may have a limited influence on the occurrence of *Alexandrium* spp. blooms, which can contaminate shellfish with PSP toxins to unsafe levels even at low cell densities (FAO, 2004). Nutrient levels may be of significance, however, together with temperature and irradiance, in determining the amplitude of blooms.

The peak of the bloom probably occurred between 15 and 22 June. After this, the structure of the water column was altered, with a temperature drop of $\sim 3\ ^{\circ}C$ and a more homogenous distribution of isohalines. Several biological processes may be responsible for bloom termination such as encystment, grazing or parasitism (Probert, 1999; Calbet et al., 2003; Figueroa et al., 2008). However, the *Alexandrium* spp. bloom collapse in summer 2006 in Cork Harbour was likely a response to a significant change in meteorological conditions (Table 3). This also coincided with higher tidal flushing, which contributed to the dilution of *Alexandrium* spp. out of the North Channel. Higher wind and tidal flushing may also have increased sediments re-suspension in the shallow parts of the North Channel, limiting thereby light penetration and growth of phototrophic phytoplankton (Ní Rathaille, 2007).

4.3. Patchy distribution: life cycle and dispersion implications

Although the timing of the maximum concentrations of *A. tamarense* and *A. minutum* did coincide, the distribution of the species was more often than not scattered across the length of the North Channel. Laboratory-based experiments conducted with resting cysts and strains of both species have shown different responses in germination success and maximum specific growth rates in relation with a temperature gradient (Ní Rathaille, 2007). However, no discernable pattern was found with respect to the distributions of both species in relation with temperature in the North Channel. The net growth rates of *A. tamarense* and *A. minutum* were comparable prior to 15 June (data not shown). Thereafter, the *A. minutum* density increased by a factor of 3.7 on 22 June whereas that of *A. tamarense* by only 1.2. This could indicate a difference in susceptibility to turbulence and/or light attenuation driven by increasing winds and tidal amplitude. Further investigations will require surveys with better resolution through fine-scale sampling.

The association of *Alexandrium* spp. patches in the vicinity of a salinity front is not uncommon in estuary areas and has been documented in several locations worldwide such as the Penze Estuary in Brittany, France, or the St. Lawrence Estuary in Canada (Probert, 1999; Larocque and Cembella, 1990). The maximum density of *A. minutum* was observed at the eastern end of the North Channel and was associated with lower surface salinity and higher nitrate concentration as has been observed previously (Touzet et al., 2008). This apparent localization may result from a physical mechanism of horizontal shear associated with an estuarine front as freshwater flows from Midleton through the North Channel and out via the East Passage on an ebb tide. Such a mechanism may promote vegetative cell encounters and favor mating events, possibly allowing the partial regeneration of cyst beds even when environmental conditions do not facilitate the development of high-density populations.

4.4. Nutrient availability and PSP toxin synthesis

Variations in nitrogen and phosphorus concentrations in cultures have had impacts on PSP toxin synthesis (Chang and McClean, 1997; Parkhill and Cembella, 1999; Wang and Hsieh, 2002). Several studies have shown phosphorus limitation to increase internal PSP toxin quotas in *Alexandrium* spp. batch cultures several fold (Boyer et al., 1987; Anderson et al., 1990; Bechemin et al., 1999; Guisande et al., 2002; Lippemeier et al., 2003; Touzet et al., 2007). In particular, Maestrini et al. (2000) showed a linear relationship between PSP toxin quotas and N:P ratios in *A. minutum*. Support for these laboratory-generated results was provided recently with a natural population of *A. minutum* from Cork Harbour (Touzet et al., 2008). However, despite its statistical significance, this relationship was less supported in the present study. This suggests more complex interactions in the mechanism controlling PSP toxin synthesis, and consideration of the evolution of internal pools of nitrogen and phosphorus in *A. minutum* may prove essential.

The internal PSP toxin quota was on average ~ 13 fmol $cell^{-1}$ and GTX3 was the dominant variant at all stages ($\sim 70\%$) between 15 and 29 June. These results are similar to those obtained from other laboratory-based and field studies in the region (Touzet, 2006). However, C-toxins, which had not been observed before in Cork Harbour, were detected on several occasions in samples. This suggests that these toxins may be synthesized under specific environmental conditions, or else there could be a subpopulation of *A. minutum* that produces C-toxins in addition to GTX2–3. The use of mass spectrometry for confirming the presence of known PSP toxin variants or characterizing new ones may be necessary to identify population markers based on toxin profile composition. The significance of relationships between geographically separated *A. minutum* populations based on toxin profiles should also be examined by using molecular fingerprinting methods such as AFLP or microsatellite markers (John et al., 2004; Nagai et al., 2006).

5. Summary

A mixed *Alexandrium* spp. bloom of moderate intensity occurred in June 2006 in the North Channel area of Cork Harbour. The distributions of *A. minutum* and *A. tamarense* were investigated using taxa-specific FISH probes throughout the duration of the bloom. The distributions of both species often proved heterogeneous and a high concentration *A. minutum* cell aggregate was detected at the east end of the North Channel at the peak of the bloom. Cell concentrations were on average above $\sim 3 \times 10^4$ cells L^{-1} for at least 14 days and the bloom termination coincided

with unfavorable meteorological conditions and a spring tide. Monitoring of toxic *Alexandrium* species in Ireland will require the use of molecular methods for reliable identification and quantification. Future investigations will focus on resolving the dynamics of the formation, maintenance and dispersion of cell aggregates near freshwater/marine interfaces through high-resolution sampling for the optimized parameterization of predictive models.

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References

Andersen, P., Enevoldsen, H., Anderson, D.M., 2003. Harmful algal monitoring programme and action plan design. In: Hallegraeff, G.M., Anderson, D.M., Cembella, A.D. (Eds.), Manual on Harmful Marine Microalgae. UNESCO, Paris, pp. 627–647.

Anderson, D.M., 1998. Physiology and bloom dynamics of toxic *Alexandrium* species with emphasis on life cycle transitions. In: In: Anderson, D.M., Cembella, A.D., Hallegraeff, G.M. (Eds.), Physiological Ecology of Harmful Algal Blooms. NATO AS1 Series,, vol. G41. Springer-Verlag, Berlin, Heidelberg, pp. 29–48.

Anderson, D.M., 1989. Toxic algal blooms and red tides: a global perspective. In: Okaichi, T., Anderson, D.M., Nemoto, T. (Eds.), Red Tides: Biology, Environmental Science and Toxicology. Elsevier, New York, pp. 11–16.

Anderson, D.M., Keafer, B.A., McGillicuddy, D.J., Mickelson, M.J., Keay, K.E., Libby, P.S., Manning, J.P., Mayo, C.A., Whittaker, D.K., Hickey, J.M., He, R., Lynch, D.R., Smith, K.W., 2005a. Initial observations of the 2005 *Alexandrium fundyense* bloom in southern New England: general patterns and mechanisms. Deep-Sea Research II 52 (19–21), 2856–2876.

Anderson, D.M., Kulis, D., Keafer, B.A., Gribble, K.E., Marin, R., Scholin, C.A., 2005b. Identification and enumeration of *Alexandrium* spp from the Gulf of Maine using molecular probes. Deep-Sea Research II 52 (19–21), 2467–2490.

Anderson, D.M., Kulis, D.M., Sullivan, J.J., Hall, S., Lee, C., 1990. Dynamics and physiology of saxitoxin production by the dinoflagellates *Alexandrium* spp. Marine Biology 104, 511–524.

Balech, E., 1995. In: The Genus Alexandrium Halim (Dinoflagellata). Sherkin Island Marine Station, Sherkin Island Co Cork, Ireland.

Bechemin, C., Grzebyk, D., Hachame, F., Hummert, C., Maestrini, S.Y., 1999. Effect of different nitrogen/phosphorus nutrient ratios on the toxin content in *Alexandrium minutum*. Aquatic Microbial Ecology 20 (2), 157–165.

Boyer, G.L., Sullivan, J.J., Andersen, R.J., Harrison, P.J., Taylor, F.J.R., 1987. Effects of nutrient limitation on toxin production and composition in the marine dinoflagellate *Protogonyaulax tamarensis*. Marine Biology 96 (1), 123–128.

Calbet, A., Vaque, D., Felipe, J., Vila, M., Monserrat Sala, M., Alcaraz, M., Estrada, M., 2003. Relative grazing impact of microzooplankton and mesozooplankton on a bloom of the toxic dinoflagellate *Alexandrium minutum*. Marine Ecology Progress Series 259, 303–309.

Cembella, A.D., 1998. Ecophysiology and metabolism of paralytic shellfish toxins in marine microalgae. In: Anderson, D.M., Cembella, A.D., Hallegraeff, G.M. (Eds.), Physiological Ecology of Harmful Algal Blooms. NATO ASI Series, vol. G41. Springer-Verlag, Berlin, Heidelberg, pp. 381–403.

Chang, F.H., McClean, M., 1997. Growth response of *Alexandrium minutum* (Dinophyceae) as a function of three different nitrogen sources and irradiance. New Zealand Journal of Marine and Freshwater Research 31, 1–7.

Diercks, S., Medlin, L.K., Metfies, K., 2008. Colorimetric detection of the toxic dinoflagellate *Alexandrium minutum* using sandwich hybridization in a microtiter plate assay. Harmful Algae 7 (2), 137–145.

Dyhrman, S.T., Erdner, D., Du, J.L., Galac, M., Anderson, D.M., 2006. Molecular quantification of toxic *Alexandrium fundyense* in the Gulf of Maine using real-time PCR. Harmful Algae 5 (3), 242–250.

Edvardsen, B., Shalchian-Tabrizi, K., Jakobsen, K.S., Medlin, L., Dahl, E., Brubak, S., Paasche, E., 2003. Genetic variability and molecular phylogeny of *Dinophysis* species (Dinophyceae) from Norwegian waters inferred from single cell analyses of rDNA. Journal of Phycology 39 (2), 395–408.

Erdner, D.L., L., Percy, Lewis, J., Anderson, D.M., 2010. A quantitative real-time PCR assay for the identification and enumeration of *Alexandrium* cysts in marine sediments. Deep Sea Research II 57(3–4), 279–287.

FAO, 2004. Marine Biotoxins FAO Food and Nutrition. Paper 80 Food and Agriculture, Organization of the United Nations, Rome.

Figueroa, R.I., Garcés, E., Massana, R., Camp, J., 2008. Description, host-specificity, and strain selectivity of the dinoflagellate parasite *Parvilucifera sinerae* sp. nov. (Perkinsozoa). Protist 159, 563–578.

Fritz, L., Triemer, R.E., 1985. A rapid simple technique utilizing Calcofluor White M2R for the visualization of dinoflagellate thecal plates. Journal of Phycology 21, 662–664.

Galluzzi, L., Penna, A., Bertozzini, E., Vila, M., Garces, E., Magnani, M., 2004. Development of a real-time PCR assay for rapid detection and quantification of *Alexandrium minutum* (a dinoflagellate). Applied and Environmental Microbiology 70 (2), 1199–1206.

Gribble, K.E., Keafer, B.A., Quilliam, M.A., Cembella, A.D., Kulis, D.M., Manahan, A., Anderson, D.M., 2005. Distribution and toxicity of *Alexandrium ostenfeldii* (Dinophyceae) in the Gulf of Maine USA. Deep-Sea Research II 52 (19–21), 2745–2763.

Guisande, C., Frangopulos, M., Maneiro, I., Vergara, A.R., Riveiro, I., 2002. Ecological advantages of toxin production by the dinoflagellate *Alexandrium minutum* under phosphorus limitation. Marine Ecology Progress Series 225, 169–176.

Hallegraeff, G.M., 2003. Harmful algal blooms: a global overview. In: Hallegraeff, G.M., Anderson, D.M., Cembella, A.D. (Eds.), Manual on Harmful Marine Microalgae. UNESCO, Paris, pp. 25–49.

Hallegraeff, G.M., Munday, B.L., Baden, D.G., Whitney, P.L., 1998. Chattonella marina raphidophyte bloom associated with mortality of cultured bluefin tuna (*Thunnus maccoyii*). in South Australia. In: Reguera, B., Blanco, J., Fernandez, M.L., Wyatt, T. (Eds.), Harmful Algae. Xunta de Galicia and IOC of UNESCO, Santiago de Compostela, Spain, pp. 93–96.

Hansen, G., Daugbjerg, N., Henriksen, P., 2000. Comparative study of *Gymnodinium mikimotoi* and *Gymnodinium aureolum* comb nov (=*Gyrodinium aureolum*) based on morphology pigment composition and molecular data. Journal of Phycology 36 (2), 394–410.

Hosoi-Tanabe, S., Sako, Y., 2005. Species-specific detection and quantification of toxic marine dinoflagellates *Alexandrium tamarense* and *A. catenella* by real-time PCR assay. Marine Biotechnology 7 (5), 506–514.

John, U., Cembella, A., Hummert, C., Elbrachter, M., Groben, R., Medlin, L., 2003. Discrimination of the toxigenic dinoflagellates *Alexandrium tamarense* and *A. ostenfeldii* in co-occurring natural populations from Scottish coastal waters. European Journal of Phycology 38 (1), 25–40.

John, U., Groben, R., Beszteri, B., Medlin, L., 2004. Utility of amplified fragment length polymorphisms (AFLP) to analyze genetic structures within the *Alexandrium tamarense* species complex. Protist 155 (2), 169–179.

John, U., Medlin, L.K., Groben, R., 2005. Development of specific rRNA probes to distinguish between geographic clades of the *Alexandrium tamarense* species complex. Journal of Plankton Research 27 (2), 199–204.

Kao, C.Y., 1993. Paralytic shellfish poisons. In: Falconer, I.R. (Ed.), Algal Toxins in Seafood and Drinking Waters. Academic Press, London, pp. 75–86.

Larocque, R., Cembella, A.D., 1990. Ecological parameters associated with the seasonal occurrence of *Alexandrium* spp. and consequent shellfish toxicity in the lower St. Lawrence Estuary (Eastern Canada). In: Graneli, E., Sundstrom, B., Edler, L., Anderson, D.M. (Eds.), Toxic Marine Phytoplankton. Elsevier, New York, pp. 368–373.

Lazerges, M., Perrot, H., Antoine, E., Defontaine, A., Compere, C., 2006. Oligonucleotide quartz crystal microbalance sensor for the microalgae *Alexandrium minutum* (Dinophyceae). Biosensors and Bioelectronics 21 (7), 1355–1358.

Lilly, E.L., Halanych, K.M., Anderson, D.M., 2005. Phylogeny biogeography and species boundaries within the *Alexandrium minutum* group. Harmful Algae 4 (6), 1004–1020.

Lilly, E.L., Halanych, K.M., Anderson, D.M., 2007. Species boundaries and global biogeography of the *Alexandrium tamarense* complex (Dinophyceae). Journal of Phycology 43 (6), 1329–1338.

Lippemeier, S., Frampton, D.M.F., Blackburn, S.I., Geier, S.C., Negri, A.P., 2003. Influence of phosphorus limitation on toxicity and photosynthesis of *Alexandrium minutum* (Dinophyceae) monitored by in-line detection of variable chlorophyll fluorescence. Journal of Phycology 39 (2), 320–331.

Luckas, B., Hummert, C., Oshima, Y., 2003. Analytical methods for paralytic shellfish poisons. In: Hallegraeff, G.M., Anderson, D.M., Cembella, A.D. (Eds.), Manual on Harmful Marine Microalgae. UNESCO, Paris, pp. 191–209.

McDonald, S.M., Sarno, D., Zingone, A., 2007. Identifying *Pseudo-nitzschia* species in natural samples using genus-specific PCR primers and clone libraries. Harmful Algae 6 (6), 849–860.

McGillicuddy, D.J., Anderson, D.M., Lynch, D.R., Townsend, D.W., 2005. Mechanisms regulating large-scale seasonal fluctuations in *Alexandrium fundyense* populations in the Gulf of Maine: results from a physical–biological model. Deep-Sea Research II 52 (19–21), 2698–2714.

Maestrini, S.Y., Bechemin, C., Grzebyk, D., Hummert, C., 2000. Phosphorus limitation might promote more toxin content in the marine invader dinoflagellate *Alexandrium minutum*. Plankton Biology and Ecology 47, 7–11.

Miller, P.E., Scholin, C.A., 1998. Identification and enumeration of cultured and wild *Pseudo-nitzschia* (Bacillariophyceae) using species-specific LSU rRNA-targeted fluorescent probes and filter-based whole cell hybridization. Journal of Phycology 34, 371–382.

Moorthi, S.D., Countway, P.D., Stauffer, B.A., Caron, D.A., 2006. Use of quantitative real-time PCR to investigate the dynamics of the red tide dinoflagellate *Lingulodinium polyedrum*. Microbial Ecology 52 (1), 136–150.

Nagai, S., McCauley, L., Yasuda, N., Erdner, D.L., Kulis, D.M., Matsuyama, Y., Itakura, S., Anderson, D.M., 2006. Development of microsatelliate markers in the toxic dinoflagellate *Alexandrium minutum* (Dinophyceae). Molecular Ecology Notes 6 (3), 756–758.

Ní Rathaille, A., 2007. Modelling *Alexandrium* bloom dynamics in Cork Harbour, Ireland. Ph.D. Thesis, National University of Ireland, Galway.
Ní Rathaille, A., Touzet, N., Raine, R., 2008. Factors controlling *Alexandrium* spp. bloom dynamics in Cork Harbour Ireland. In: Proceedings of the Sixth International Conference on Molluscan Shellfish Safety, Blenheim, New Zealand.
Oshima, Y., 1995. Postcolumn derivatization liquid chromatographic method for paralytic shellfish toxins. Journal of AOAC International 78, 528–532.
Parkhill, J.P., Cembella, A.D., 1999. Effects of salinity, light and inorganic nitrogen on growth and toxigenicity of the marine dinoflagellate *Alexandrium tamarense* from northeastern Canada. Journal of Plankton Research 21 (5), 939–955.
Probert, I., 1999. Sexual reproduction and ecophysiology of the marine dinoflagellate *Alexandrium minutum* Halim. Ph.D. Thesis, University of Westminster, UK.
Riley, J.P., 1953. The spectrophotometric determination of ammonia in natural waters with particular reference to sea-water. Analytica Chimica Acta 9, 575–589.
Sanz, J.L., Köchling, T., 2007. Molecular biology techniques used in wastewater treatment: an overview. Process Biochemistry 42, 119–133.
Scholin, C.A., Anderson, D.M., 1996. LSU rDNA-based RFLP assays for discriminating species and strains of *Alexandrium*. Journal of Phycology 32, 1022–1035.
Scholin, C.A., Hallegraeff, G.M., Anderson, D.M., 1995. Molecular evolution of the *Alexandrium tamarense* species complex (Dinophyceae): dispersal in the North American and west Pacific regions. Phycologia 34, 472–485.
Shumway, S.E., 1990. A review of the effects of algal blooms on shellfish and aquaculture. Journal of the World Aquaculture Society 21 (2), 65–104.
Smayda, T.J., 2003. Environmental monitoring, with examples from Narragansett Bay. In: Hallegraeff, G.M., Anderson, D.M., Cembella, A.D. (Eds.), Manual on Harmful Marine Microalgae. UNESCO, Paris, pp. 595–625.
Strickland, J.D.H., Parsons, T.R., 1972. In: A Practical Handbook of Seawater Analysis. The Alger Press Ltd., Ottawa.
Touzet, N., 2006. The biogeography, ecophysiology and phylogeny of *Alexandrium* Halim in Irish waters. Ph.D. Thesis, National University of Ireland, Galway.
Touzet, N., Franco, J.M., Raine, R., 2007. Influence of inorganic nutrition on growth and PSP toxin production of *Alexandrium minutum* (Dinophyceae) from Cork Harbour, Ireland. Toxicon 50 (1), 106–119.
Touzet, N., Franco, J.M., Raine, R., 2008. PSP toxin analysis and discrimination of the naturally co-occurring *Alexandrium tamarense* and *A. minutum* (Dinophyceae) in Cork Harbour Ireland. Aquatic Microbial Ecology 51 (3), 285–299.
Utermöhl, H., 1958. Zur vervollkomnung der quantitativen phytoplankton metodik. Mitteilungen Internationale Vereinigung Theoretische und Angewandte Limnologie 9, 1–38.
Vila, M., Giacobbe, M.G., Maso, M., Gangemi, E., Penna, A., Sampedro, N., Azzaro, F., Camp, J., Galluzzi, L., 2005. A comparative study on recurrent blooms of *Alexandrium minutum* in two Mediterranean coastal areas. Harmful Algae 4 (4), 673–695.
Wakeham, S.G., Amann, R., Freeman, K.H., Hopmans, E.C., Jorgensen, B.B., Putnam, I.F., Schouten, S., Sinninghe Damste, J.S., Talbot, H.M., Woebken, D., 2007. Microbial ecology of the stratified water column of the Black Sea as revealed by a comprehensive biomarker study. Organic Geochemistry 38 (12), 2070–2097.
Wang, D.-Z., Hsieh, D.P.H., 2002. Effects of nitrate and phosphate on growth and C2 toxin productivity of *Alexandrium tamarense* CI01 in culture. Marine Pollution Bulletin 45 (1-12), 286–289.
Wright, J.L.C., 1995. Dealing with seafood toxins: present approaches and future options. Food Research International 28 (4), 347–358.

II.2: Análisis comparativo de los métodos de oxidación pre- y post-columna para la detección de toxinas paralíticas.

Resumen

Las toxinas PSP son compuestos naturales altamente tóxicos producidos por dinoflagelados comúnmente presentes en el fitoplancton marino. Los mariscos contaminados con estas toxinas suponen una amenaza para la salud pública y pérdidas económicas a la industria marisquera. Por esta razón, varios métodos de HPLC se han desarrollado con el fin de obtener un mejor conocimiento de los perfiles de toxinas en los mariscos y muestras de dinoflagelados. Estos métodos han sido objeto de continuas modificaciones para mejorar y acortar el tiempo de análisis en un seguimiento de control rutinario. En este trabajo, se analizaron varias muestras por los métodos de HPLC con oxidación pre- y post-columna para comparar el perfil de toxinas. Todas las toxinas PSP fueron individualmente identificadas y cuantificadas mediante el método de oxidación post-columna. Sin embargo, aunque el método de oxidación pre-columna es significativamente más sensible y detecta cantidades más bajas de toxina, proporciona un valor total de las toxinas que co-eluyen juntas, como la GTX2 y 3, GTX1 y 4, dcGTX2 y dcGTX3. Los resultados obtenidos por ambos métodos de HPLC mostraron concentraciones de toxinas similares (expresados en µg/mL) en muestras de mejillón, sin embargo, cuando las muestras de dinoflagelados fueron analizadas, el perfil de toxinas y la concentración fueron diferentes. En resumen, el método de oxidación post-columna es preciso para determinar la cantidad de cada toxina PSP individualmente y para conocer el perfil tóxico real de una muestra. Además, es un método fácil y rápido para analizar un gran número de muestras. El método de oxidación pre-columna es más apropiado cuando se analizan muestras de mejillón aunque el tiempo de preparación de las muestras es más largo.

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Comparative analysis of pre- and post-column oxidation methods for detection of paralytic shellfish toxins

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ABSTRACT

Paralytic shellfish poisoning (PSP) toxins are highly toxic natural compounds produced by dinoflagellates commonly present in marine phytoplankton. Shellfish contaminated with these toxins create significant public health threat and economic losses to the shellfish industry. For this reason, several methods of high performance liquid chromatography (HPLC) with fluorescence detection have been developed in order to gain better knowledge of toxins profiles in shellfish and dinoflagellates samples. These methods have been subjected to continuous modifications to improve and shorten the run time of analysis in the routine monitoring control. In this paper, different samples are analyzed by pre- and post-column HPLC methods to compare toxin profiles. All PSP toxins were individually identified and quantified within the post-column oxidation method. However, although the pre-column oxidation method is significantly more sensitive and detects lower toxin levels, it provides a total amount of toxins that co-elute together, as GTX2 and 3, GTX1 and 4 and dcGTX2 and dcGTX3. The results obtained by both HPLC methods showed similar toxin concentration (expressed in μg/mL) in mussel samples, however when dinoflagellates samples were analyzed the toxin profile and concentration were different. In summary, the post-column oxidation method is accurate to determine the amount of each individual PSP toxin and to know the real toxic profile of non-transformed samples. In addition, this method is easy and faster to screen a large number of samples. The pre-column HPLC method is useful when mussel samples are analyzed even though the time required to prepare the samples is longer.

1. Introduction

Paralytic shellfish poisoning (PSP) are potent neurotoxins produced by several species of dinoflagellates such as *Alexandrium*, *Gymnodinium* and *Pyrodinium* (Kodama, 2000; Dell'Aversano et al., 2008). These toxins specifically block the excitation current in nerve and muscle cells, resulting in paralysis and other symptoms (Luckas et al., 2004). Hence, the accumulation of PSP toxins in shellfish creates a serious public health problem and affect to fisheries industry. For this reason many countries have monitoring and regulatory systems that include the routine sampling of shellfish flesh for the presence of biotoxins and the examination of water samples for the presence of toxin producing phytoplankton (Hallegraeff, 1995). The mouse bioassay (MBA), developed in 1937 to check toxicity in acidic extracts of mussels (Sommer and Meyer, 1937), is the worldwide PSP official method used in monitoring programmes to prevent human intoxication. However, several limitations, including the pH-dependant high variability and low sensitivity (Van Dolah and Ramsdell, 2001), in addition to the strong opposition to animal sacrifice, has led the search of alternative methods for PSP detection. These alternatives include immunoassays, receptor binding

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assay, cell assays (Van Dolah and Ramsdell, 2001), and several chemical methods. The most common chemical method uses a combination of high performance liquid chromatography (HPLC) with either pre- or post- column oxidation followed by fluorescence detection (Lawrence and Niedzwiadek, 2001; Thomas et al., 2006). The ability of PSP toxins to be easily converted into fluorescent derivatives has been the basis for their detection (Gago-Martinez et al., 2001). These methods have the advantage of detecting and quantifying individual PSP toxins by using standards, even though not certified material of each toxin is available (Ben-Gigirey and Villar-González, 2008). The two main methodologies used to analysis of PSP toxins involve an isocratic separation of the toxins followed by a post-column oxidation (Oshima, 1995) or a pre-column oxidation of toxins followed by a gradient separation of the oxidation products (Lawrence et al., 1995). In 2005, the Lawrence method has been adopted as an official method to detect PSP toxins and it has been recently approved by the EU for monitoring these toxins (AOAC, 2005; E.U.C, 2006). However, this method seems to be useful mainly for official PSP control in certain samples since its performance depends on the toxic profile of the sample (Ben-Gigirey et al., 2007). The major impediments to widespread use of this method are the co-elution of oxidation products and the amount of time required to process samples containing significant amounts of PSP toxins (Ben-Gigirey et al., 2007). In this sense, the post- column oxidation methods based on the Oshima method (Oshima, 1995) are suitable when a full quantification of PSP toxins is required (Franco and Fernández-Vila, 1993; Chen and Chou, 2002). Nowadays the post-column oxidations methods have been subject to continuous modifications to better and reduce of run times of analysis (Rourke et al., 2008). The pre- and post- column HPLC methods, despite the many benefits of each, which includes an increased sensitivity to low concentrations of toxins and less variability in the results, present also some drawbacks that should be resolved.

PSP toxins can be *in vitro* transformed by pH and/or temperature effect (Vale et al., 2008), in addition bivalves that accumulate the toxins can also transform them (Franco and Fernández-Vila, 1993). For these reason, the sample source and the processing procedures as well as the standard composition are important items when the toxin profile of a sample will be HPLC studied. In the present work, several samples from different dinoflagellates (non-converted) and shellfish (converted) were analyzed by pre- and post- column HPLC methods in order to compare the PSP toxins profile obtained by both methods.

2. Material and methods

2.1. Chemicals and solutions

HPLC grade methanol, acetonitrile, acetic acid, sodium hydroxide, periodic acid, disodium hydrogen phosphate (Na_2HPO_4) and hydrogen peroxide were obtained from Panreac Quimica S.A. (Barcelona, Spain). Ammonium formate was purchased from Sigma Aldrich (Spain).

Peroxide oxidant was 10% hydrogen peroxide aqueous solution. Periodate oxidant was prepared daily by mixing 5 mL each of 0.03 M periodic acid, 0.3 M ammonium formate and 0.3 M Na_2HPO_4, then adjusted pH to 8.2 with 1 M NaOH using pH meter.

Standards of PSP toxins; saxitoxin (STX), neosaxitoxin (NEO), decarbamoylsaxitoxin (dcSTX), gonyautoxins 1 and 4 (GTX1,4), gonyautoxins 2 and 3 (GTX2,3), decarbamoylgonyautoxins 2 and 3 (dcGTX2,3), GTX 5 (B1), and C1 and C2 were provided by NRC Certified Reference Material Program (Institute for Marine Biosciences, Halifax, Canada) for the identification of each toxin.

2.2. Samples preparation

Several samples (1–13) from different PSP producer dinoflagellates were used. *Alexandrium tamarense* (CCMP1598 strain) was purchased from Bigelow Laboratory for Ocean Sciences (ME, USA), and several *Alexandrium* spp. samples were obtained from the west coast of Spain. The dinoflagellates were filtered through a 10 μm mesh and the cells were re-suspended in acetic acid/ethanol (3:1). Then, the cells were ultrasound lysed (three cycles of 20 s) and centrifuged at 3000 rpm for 5 min. The supernatant was separated from the pellet, evaporated to dryness and finally dissolved in 0.03 M acetic acid.

Several toxins extracts (14–17) isolated from contaminated mussels from Galicia, Spain were used. The extracts were obtained after whole flesh extraction (ethanol/acetic acid) followed by purification through weight exclusion chromatography (Alfonso et al., 1993).

The extracts, either from dinoflagellates or mussels, were cleaned from residues in centrifuge tubes (with 0.45 μm filters) before HPLC analysis by both methods. Aliquots of 100 μL of the extracts were directly injected in a 20 μL loop in the post-column oxidation method. For analysis by pre-column oxidation method, the extracts were previously oxidized with hydrogen peroxide or periodate. For the peroxide oxidation 25 μL of 10% (w/v) aqueous H_2O_2 were added to 250 μL of 1M NaOH in a 1.5 mL vial and mixed. Then 100 μL of standard solution or sample extract were added to the vial. The reaction was allowed for 2 min at room temperature. Then 20 μL of glacial acetic acid was added to stop the reaction, the solution was mixed, and 25 μL were injected into the HPLC system. For periodate oxidation 100 μL of standard solution or sample extract were added to 100 μL of deionized water in a 1.5 mL vial. Then 500 μL of periodate oxidant were added to the vial and mixed. The solution was allowed to react at room temperature for 1 min, and then 5 μL of glacial acetic acid were added and mixed. The mixture was allowed to stand for at least 10 min at room temperature before injecting 100 μL into the HPLC system.

2.3. HPLC

2.3.1. HPLC with post-column oxidation

A modification of Oshima's (Oshima, 1995) HPLC method (Vale et al., 2008) was used to identify PSP toxins. To separate the toxins an AquaSep column, reversed-phase C8 (5 μm, 4.6 × 250 mm) from ES Industries Chromega Columns was used with different mobile phases depending on the toxin group. The mobile phase to identify the GTX toxins group (acetonitrile-free) was constituted by 2 mM

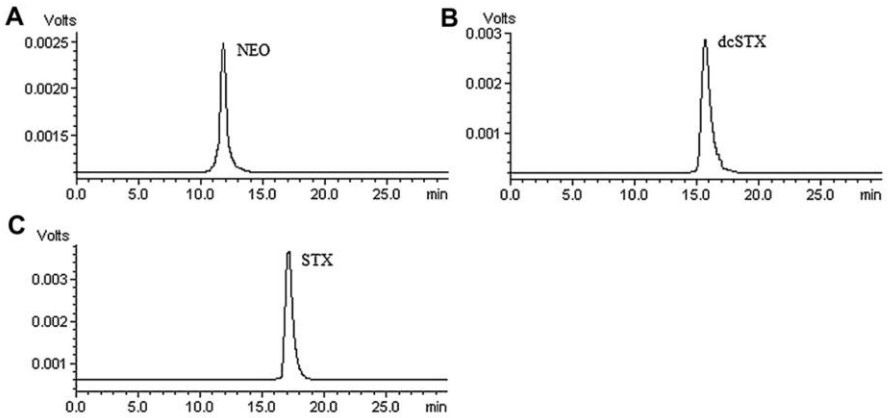


Fig. 1. Chromatograms of PSP standards from the post-column oxidation method. The toxins were diluted in 0.03 M acetic acid and then were eluted with the mobile phase that contains acetonitrile.

n-heptanesulphonic acid and 10 mM o-phosphoric acid (pH 7.2 with 1N NH_4OH). The mobile phase used to identify the STX toxins group (with acetonitrile) was made by 2 mM n-heptanesulphonic acid, 10 mM o-phosphoric acid and 10% acetonitrile (pH 7.2 with 1N NH4OH). The injection volume was 10 μL. The running time was 30 min. The flow rate was adjusted at 0.8 mL/min in a Shimadzu LC-20AD pump. A solution of 7 mM periodic acid and 50 mM sodium phosphate (pH 9.0 with 1 N NaOH) was introduced to the column elute in a T made by Teflon at a flow rate of 0.8 mL/min controlled by a Shimadzu LC-10ADvp pump. The resulting mixture was heated while passing through a teflon tube (10 m × 0.5 mm i.d.) immersed in a water bath at 70 °C. The reaction mixture was then acidified in another T with 0.5 M acetic acid (flow rate 0.1 mL/min) to reach a pH outflow of 5–7. The fluorescent eluted derivatives were monitored using a Shimadzu RF535 fluorescence detector at 340 and 410 nm excitation-emission wavelengths, respectively. The system was run through the software LC Solution from Shimadzu.

2.3.2. HPLC with pre-column oxidation

HPLC Lawrence method (Lawrence et al., 2005) involves liquid chromatography with fluorescence detection after pre-chromatographic oxidation of the PSP toxin using hydrogen peroxide for STX, GTX2 and GTX3 (combined), dcSTX, GTX5, C1 and C2 (combined) and periodate for NEO, GTX1 and GTX4 (combined). A column Supelcosil™ LC-18 (5 μm, 15 × 4.6 mm) from Sigma Aldrich was used to separate the toxins. The column oven was at 35 °C. The two mobile phases were as follows: A: 0.1M ammonium formate (pH 6 with 1M acetic acid) and B: 0.1M ammonium

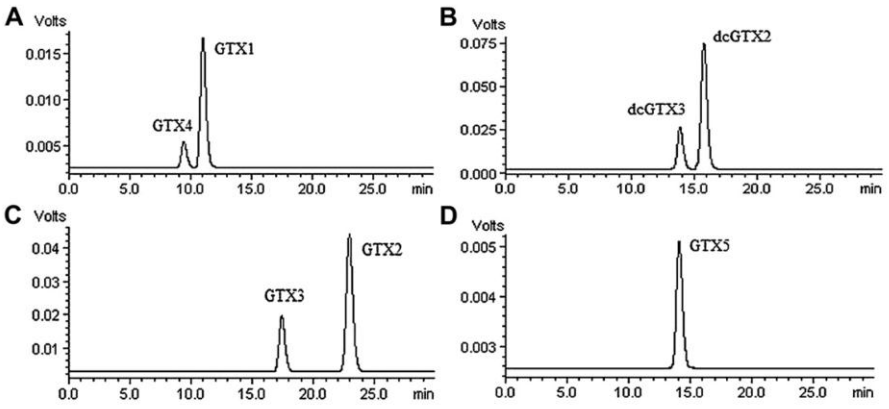


Fig. 2. Chromatograms of PSP standards from the post-column oxidation method. The toxins were diluted in 0.03 M acetic acid and then were separated with the mobile phase acetonitrile-free.

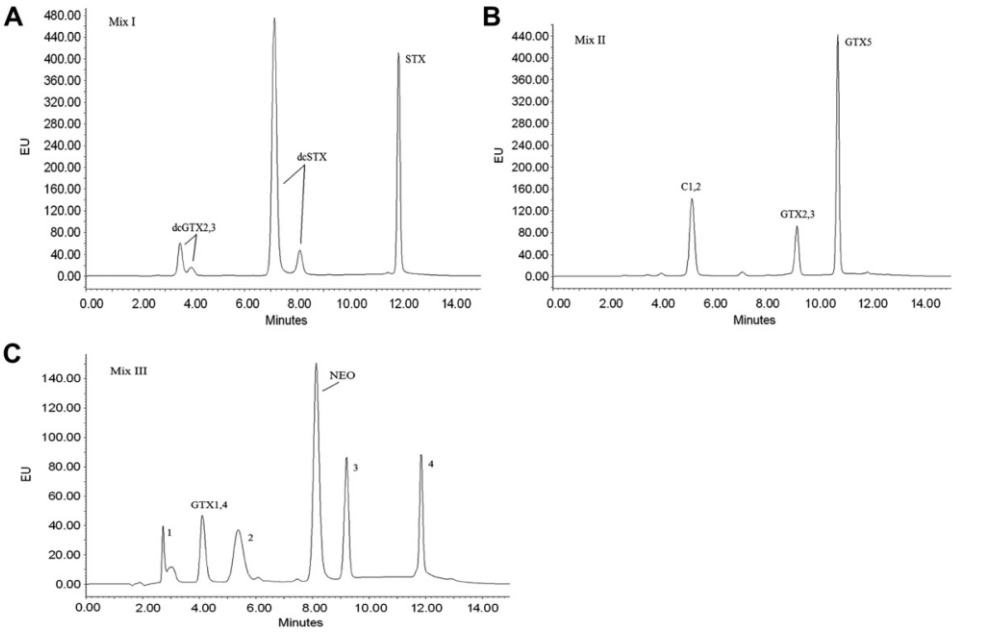


Fig. 3. Chromatograms of PSP standards mixtures from the pre-column oxidation method. (A): Mix I and (B): Mix II were injected after peroxide oxidation. (C): Mix III was injected after periodate oxidation. Peaks 1 and 2 are secondary oxidation products of GTX1,4 and peaks 3 and 4 are secondary oxidations products of NEO.

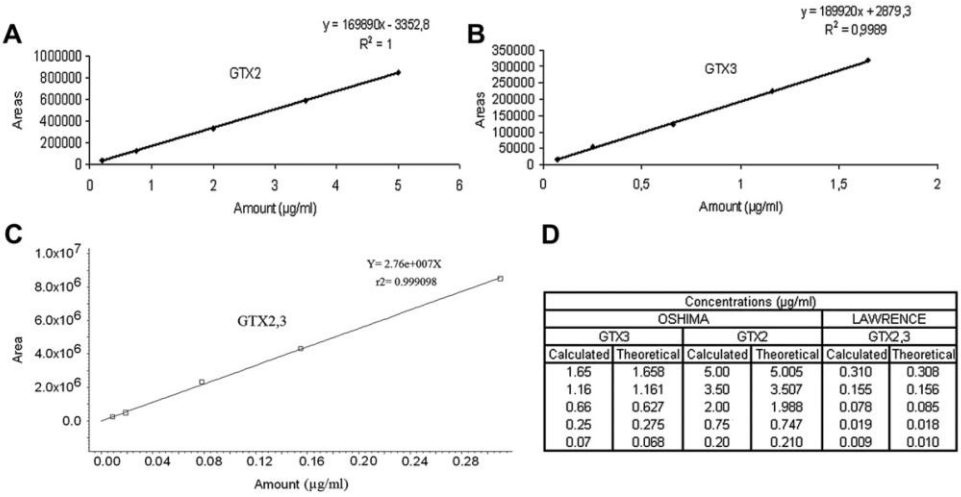
formate in 5% acetonitrile, (pH 6 1M acetic acid). The gradient program used to elute the PSP oxidation products was 0–5% mobile phase B in the first 5 min, 5–70% B for the next 4 min and back to 0% B over the next 2 min; then at 0% B for another 3 min before the next injection. The gradient was produced in a Waters 515 HPLC pump. The injection volume was 25 μL for samples oxidized with peroxide or 100 μL for samples oxidized with periodate. The flow rate was set at 1 mL/min (Ben-Gigirey et al., 2007). Detection was monitored using a Waters 2475 fluorescence detector, with excitation set to 340 nm and emission to 395 nm. The system was run through the software Empower from Waters.

2.4. Toxin identification and quantification

For determination of the PSP toxin amounts with the post-column oxidation method, a calibration curve to each toxin was done with at least five points by dilution of standards from Canada with 0.03 M acetic acid. In the pre-column oxidation method, standards from Canada were combined into 3 mixtures by appropriate dilution of standard solutions in water (dilution 1/10). For determination of PSP toxins a calibration curve with 5 points for each mixture (mix I, II and III) was done with the corresponding dilutions in water. The calibration solutions were prepared fresh daily for every series of analysis. Identification of PSP

Table 1
Retention times of PSP toxins obtained within the post- and pre- column oxidation methods.

Post-column method			Pre-column method		
Mobile phases	Toxins	RT (min)	Oxidation reactions	Toxins	RT (min)
With acetonitrile	NEO	11.7	Periodate	GTX1,4	2.7–4.1–9.2
	dcSTX	15.5		NEO	5.2–8.1–11.8
	STX	17.1			
Acetonitrile-free	GTX4	9.6	Hydrogen peroxide	dcGTX2,3	3.5–4.1
	GTX1	11.2		C1,2	5.2
	dcGTX3	13.7		dcSTX	7.1–8.1
	GTX5	14.1		GTX2,3	9.2
	dcGTX2	15.7		GTX5	10.7
	GTX3	17.6		STX	11.8
	GTX2	23.2			



Concentrations (µg/ml)					
OSHIMA				LAWRENCE	
GTX3		GTX2		GTX2,3	
Calculated	Theoretical	Calculated	Theoretical	Calculated	Theoretical
1.65	1.658	5.00	5.005	0.310	0.308
1.16	1.161	3.50	3.507	0.155	0.156
0.66	0.627	2.00	1.988	0.078	0.085
0.25	0.275	0.75	0.747	0.019	0.018
0.07	0.068	0.20	0.210	0.009	0.010

Fig. 4. Calibration curves of GTX2 (A) and GTX3 (B) from the post-column oxidation method and GTX2,3 from the pre-column oxidation method (C). GTX 2 and GTX3 concentrations in µg/mL used in the calibration curves (D).

toxins was done comparing retention times and the number of oxidation products of the samples with that of the standard substances. Each PSP toxin was quantitatively determined by direct comparison of peak areas in the test samples with those of the standards using the corresponding calibration curves.

Results are shown expressed as the mean ± SEM.

3. Results

The aim of this paper is the identification and quantification of PSP toxins in several samples by pre- and post-column HPLC methods.

In the post-column oxidation method, the standards of PSP toxins were diluted in 0.03 M acetic acid and then individually injected into the system. First, standards of NEO, dcSTX and STX were injected using the mobile phase that contains acetonitrile. As shown in Fig. 1, only one peak from each PSP toxin is detected. NEO was eluted at 11.7 min (Fig. 1A), dcSTX at 15.5 min (Fig. 1B) and STX at 17.1 min (Fig. 1C). Then GTX1,4; dcGTX2,3; GTX2,3 and GTX5 were injected with the acetonitrile-free mobile phase. As shown in Fig. 2, GTX1,4 produces a first peak identified as GTX4 in the retention time of 9.6 min and a second peak identified as GTX1 in the retention time of 11.2 min (Fig. 2A). When dcGTX2,3 are injected, two peaks are eluted at retention times of 13.7 and 15.7 min that correspond to dcGTX3 and dcGTX2 respectively (Fig. 2B). GTX2,3 also produce two peaks, the first peak identified as GTX3 (17.6 min) and the second peak identified as GTX2 (23.2 min) (Fig. 2C). Finally when GTX5 was injected, one peak was eluted within 14.1 min (Fig. 2D).

In the pre-column method, the standards solutions were combined into three mixtures (Mix I, II and III) and diluted with deionized water. For the present assay, Mix I was formed by dcGTX2,3, dcSTX and STX; Mix II was done by C1,2, GTX2,3 and GTX5 and the Mix III was constituted by GTX1,4 and NEO. Fig. 3 shows the chromatograms of these mixtures injected after peroxide, Fig. 3A and B, or periodate, Fig. 3C, oxidation. As Fig. 3A and B shown, STX (11.8 min), GTX2,3 (9.2 min), GTX5 (11.7 min) and C1,2 (5.2 min) produce only one peak after peroxide oxidation, while with the same oxidation solution dcGTX2,3 and dcSTX produce two peaks (two oxidation products). In this case, only the first eluting peaks at 3.5 and 7.1 min respectively are used for quantification (Fig. 3A). NEO and GTX1,4, mix III, produce three peaks after periodate oxidation but only the second eluting peaks at 4.1 min for GTX1,4 and 8.1 min for NEO are used for quantification (Fig. 3C), the other peaks are secondary oxidation products of both toxins. The retention times of PSP toxins obtained with both methods are summarized in Table 1.

To quantify the toxins in samples, several concentrations of each PSP standard were injected to obtain

Table 2
Limits of quantification (LOQ) and limits of detection (LOD) for each toxin (expressed in ng/mL) calculated in the pre- and post- column oxidation methods.

Toxins	Post-column method		Pre-column method	
	LOQ	LOD	LOQ	LOD
NEO	250	90	8	1.2
dcSTX	60	20	6	0.2
STX	90	30	7	0.4
GTX1	90	30	9	3
GTX4	150	50		
dcGTX2	20	7	8	2.7
dcGTX3	20	6		
GTX5	150	50	8	0.4
GTX2	30	9	9	1.5
GTX3	30	8		

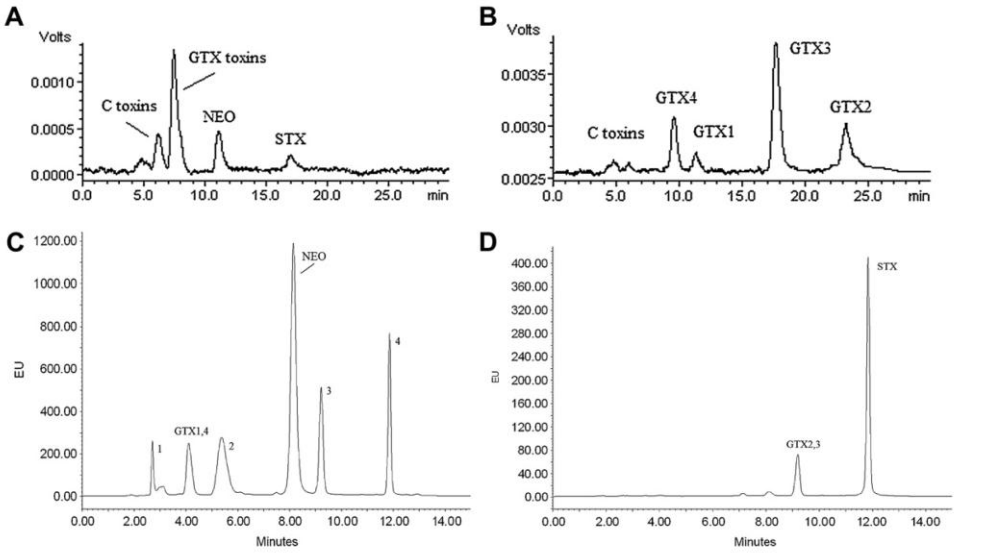


Fig. 5. Toxin profile of sample 1 (from dinoflagellates) from the post-column oxidation method after the analysis with the mobile phase with acetonitrile (A) and the mobile phase acetonitrile-free (B) and from the pre-column oxidation method after periodate oxidation (C) and peroxide oxidation (D).

calibration curves with good correlation coefficients ($R > 0.96$). The Fig. 4 shows the GTX2,3 calibration curves achieved by both HPLC methods. In the post-column oxidation method two calibration curves, one for GTX2 (Fig. 4A) and other for GTX3 (Fig. 4B) were obtained. However in the pre-column oxidation method only one calibration curve is possible to those toxins that co-elute together, as GTX2,3 (Fig. 4C). From the calibration curves obtained with each PSP standard, the limits of quantification (LOQ) and detection (LOD) were calculated in both pre- and post-column oxidation methods. As it is shown in Table 2, the LOQ in the pre-column method is in the range of 6–9 ng/mL and the LOD in the range of 0.4–3 ng/mL. In the post-column method, this limits are higher, LOQ is between 20 and 250 ng/mL and the LOD between 6 and 90 ng/mL.

Then several samples of dinoflagellates (13) and mussel (4) were analyzed. Fig. 5 shows the chromatograms of sample 1 (from dinoflagellates). In a first isocratic elution with the mobile phase with acetonitrile, the toxins NEO and STX were separated and quantified; in addition C and GTX group toxins were also detected (Fig. 5A). Then, in a second isocratic run with the acetonitrile-free mobile phase, the toxins GTX1, GTX2, GTX3 and GTX4 were separated and quantified (Fig. 5B). Afterward the sample was re-analyzed by the pre-column oxidation method. When the sample was oxidized with periodate, three peaks corresponding to GTX1,4 and three peaks corresponding to NEO were detected (Fig. 5C). Only the peaks eluted within 4.1 and 8.1 min were used to quantify GTX1,4 and NEO respectively. Then, the sample was oxidized with peroxide and the presence of GTX2,3 (9.2 min) and STX (11.8 min) was confirmed (Fig. 5D). The PSP concentrations values obtained in the sample by the post-column oxidation method were 0.09 ± 0.01 μg/mL of GTX2, 0.22 ± 0.03 μg/mL of GTX3, 0.11 ± 0.02 μg/mL of GTX1, 0.50 ± 0.03 μg/mL of GTX4, 0.47 ± 0.02 μg/mL of STX and 2.37 ± 0.15 μg/mL of NEO. The pre-column oxidation method provided amounts of 0.27 ± 0.03 μg/mL of GTX2,3, 1.40 ± 0.10 μg/mL of GTX1,4, 0.32 ± 0.02 μg/mL of STX and 2.26 ± 0.06 μg/mL of NEO. In the pre-column oxidation method the amount of GTX 2, 3, 1 and 4 can be calculated by using the percentage of each toxin present in the standard, that is 0.18 μg/mL of GTX2, 0.090 μg/mL of GTX3, 0.94 μg/mL of GTX1 and 0.46 μg/mL of GTX4. Fig. 6 shows the toxin profile of sample 14 (mussel extract). Fig. 6A and B show chromatograms obtained within the post-column oxidation method and Fig. 6C shows the chromatogram obtained after peroxide oxidation within the pre-column oxidation method. The amount of toxins obtained in the first case were 0.56 ± 0.04 μg/mL of STX, 10.00 ± 0.04 μg/mL of GTX5, 2.81 ± 0.14 μg/mL of dcGTX2, 1.23 ± 0.15 μg/mL of GTX2 and 0.87 ± 0.06 μg/mL of GTX3. The PSP concentrations obtained in the second case were 0.65 ± 0.03 μg/mL of STX, 9.78 ± 0.28 μg/mL of GTX5, 3.37 ± 0.24 μg/mL of dcGTX2,3 and 1.98 ± 0.15 μg/mL of GTX2,3. From these data the amount of GTX2, GTX3, dcGTX2 and dcGTX3 can be calculated by using the percentage of each toxin present in the standard, that is 1.32 μg/mL of GTX2, 0.65 μg/mL of GTX3, 2.42 μg/mL of dcGTX2 and 0.95 μg/mL of dcGTX3. The results of the quantification of each PSP toxin in the samples obtained either by post- and pre- column oxidation methods are summarized in Tables 3 and 4. In summary, independently of the HPLC method used the amount of toxins reported in converted samples (mussels) are similar, however the quantification of GTX2, GTX3, GTX1, GTX4, dcGTX2 and dcGTX3 by using the pre-column

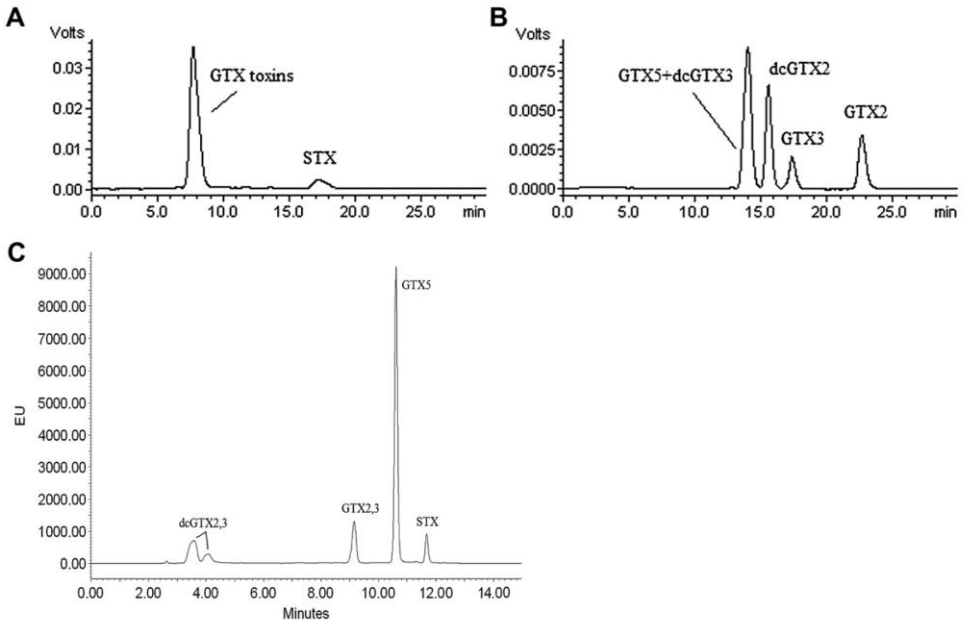


Fig. 6. Toxin profile of sample 14 (from mussel extract) from the post-column oxidation method after analyses with the mobile phase with acetonitrile (A) and the mobile phase acetonitrile-free (B) and from the pre-column oxidation method after peroxide oxidation (C).

oxidation method is not properly done when non-transformed samples (dinoflagellates) are analyzed.

4. Discussion

Several authors have quantitatively compared the detection of PSP toxins by MBA and HPLC (Lawrence and Oshima). They found a linear relationship between both techniques. Although both methods have been subjected to continuous modifications, still exist some advantages and disadvantages that make them difficult to employ as official methods in monitoring programs.

The Oshima method (Oshima, 1995) has recently become quite popular because it can chromatographically separate all PSP toxins. However, it is not a very applicable method for shellfish surveillance in monitoring situations, because it involves the need to perform three time consuming separate runs to perceive the presence of all toxins, even though this problem was been almost solved (Rourke et al., 2008). The pre-column oxidation method proposed by Lawrence and Menard (Lawrence and Menard, 1991a) and Lawrence et al. (Lawrence et al., 1991b, 1995, 1996), is also very much time consuming and does not separate all toxins, but it is slightly more sensitive that the Oshima method. This is especially important at levels of food safety, because samples that contain lower amounts of toxins can be verified by the Lawrence method, at concentrations approximately 10 times below the LOD of Oshima. However Lawrence method is not easy to implement at a daily routine basis because of drawbacks in employing the method (Ben-Gigirey et al., 2007). These methods are used as an alternative to mouse bioassay for monitoring of PSP levels in shellfish. However, nowadays the detection of toxins in dinoflagellates cultures, seawater samples or even adsorbed onto resins are becoming more frequent. The toxin profiles in these samples are different of the profile observed in shellfish samples because the toxins are not converted or transformed. Therefore it is very important to use an identification method that provides a full quantification of all PSP toxins in any type of sample.

In the pre-column oxidation method PSP toxins yield highly fluorescent derivatives with both peroxide and periodate oxidations (Lawrence et al., 1996), that can incriminate the co-elution of different peaks during the periodate oxidation, which leads to inadequate identification and quantification of several PSP toxins (Ben-Gigirey et al., 2007). The advantage of the post-column oxidation method is that there is no overlapping of peaks and all toxins can be properly separated. In addition in this method each toxin produce only one peak after oxidation, while in the pre-column oxidation method some toxins (NEO, GTX1,4…) produce more oxidation products that co-eluted in the same retention time than other toxins.

The post-column method implicates the use of two mobile phases to separate STX and GTX toxins groups. Although in a first run with the mobile phase with acetonitrile the presence of NEO, dcSTX and STX can be determined and also consequently exhibits the presence of Cs and GTX toxins (Franco and Fernández-Vila, 1993). This issue could be compared with the periodate oxidation of

Table 3
PSP toxins concentrations (μg/mL) in the samples obtained by HPLC with the post-column oxidation method. Mean ± SEM of three injections of the samples.

Post-column method												
Samples	GTX1	GTX4	Total (1 + 4)	GTX2	GTX3	Total (2 + 3)	dcGTX2	dcGTX3	Total dc(2 + 3)	GTX5	STX	NEO
1	0.11 ± 0.02	0.50 ± 0.03	0.61	0.09 ± 0.01	0.22 ± 0.03	0.31					0.47 ± 0.02	2.37 ± 0.15
2				0.16 ± 0.02	0.25 ± 0.02	0.41						
3				<LOQ	0.13 ± 0.02	0.13						
4				<LOQ	0.03 ± 0.01	0.03						
5				<LOQ	<LOQ							
6	0.20 ± 0.02	0.93 ± 0.05	1.13	–	0.13 ± 0.03	0.13					0.35 ± 0.05	0.34 ± 0.03
7				0.06 ± 0.01	0.17 ± 0.02	0.23						
8				0.03 ± 0.01	0.12 ± 0.02	0.15						
9	–	0.28 ± 0.02	0.28	–	0.40 ± 0.01	0.40					5.99 ± 0.5	0.74 ± 0.02
10				0.10 ± 0.01	0.25 ± 0.02	0.35						
11				0.11 ± 0.01	0.31 ± 0.02	0.42						
12				–	0.04 ± 0.01	0.04						
13				0.08 ± 0.01	0.22 ± 0.02	0.30						
14				1.23 ± 0.15	0.87 ± 0.06	2.10	2.81 ± 0.14	–	2.81	10.00 ± 0.04	0.56 ± 0.04	
15				1.64 ± 0.19	1.16 ± 0.07	2.80	3.74 ± 0.18	0.61 ± 0.04	4.35		0.74 ± 0.05	
16				0.88 ± 0.11	0.62 ± 0.04	1.50	2.00 ± 0.01	0.33 ± 0.03	2.33	7.12 ± 0.10	0.40 ± 0.02	
17				1.32 ± 0.12	0.93 ± 0.06	2.25	2.98 ± 0.04	0.49 ± 0.02	3.47		0.60 ± 0.03	

Table 4
PSP toxins concentrations (μg/mL) in the samples obtained by HPLC with the pre-column oxidation method. Mean ± SEM of three injections of the samples.

Pre- Column method												
Samples	GTX2,3	GTX2[a]	GTX3[a]	GTX1,4	GTX1[a]	GTX4[a]	dcGTX2,3	dcGTX2[a]	dcGTX3[a]	GTX5	STX	NEO
1	0.27 ± 0.03	0.18	0.09	1.40 ± 0.10	0.94	0.46					0.32 ± 0.02	2.26 ± 0.06
2	0.44 ± 0.02	0.29	0.14									
3	0.07 ± 0.02	0.05	0.02									
4	0.02 ± 0.01	0.01	<LOQ									
5	0.02 ± 0.01	0.01	<LOQ									
6	0.10 ± 0.01	0.07	0.03	1.76 ± 0.05	1.18	0.58					0.17 ± 0.02	0.36 ± 0.04
7	0.11 ± 0.03	0.07	0.04									
8	0.06 ± 0.02	0.04	0.02									
9	0.37 ± 0.02	0.25	0.12	0.93 ± 0.02	0.62	0.31					4.24 ± 0.3	0.81 ± 0.05
10	0.21 ± 0.05	0.14	0.07									
11	0.19 ± 0.03	0.13	0.06									
12	0.03 ± 0.01	0.02	0.01									
13	0.20 ± 0.03	0.13	0.07									
14	1.98 ± 0.15	1.32	0.65				3.37 ± 0.24	2.42	0.95	9.78 ± 0.28	0.65 ± 0.03	
15	2.63 ± 0.19	1.76	0.87				4.48 ± 0.20	3.22	1.26		0.86 ± 0.03	
16	1.41 ± 0.11	0.94	0.46				2.39 ± 0.15	1.72	0.67	6.84 ± 0.18	0.47 ± 0.02	
17	2.11 ± 0.08	1.41	0.70				3.58 ± 0.20	2.57	1.01		0.71 ± 0.03	

[a] Concentrations calculated based on standards manufacturer information.

Lawrence method (Lawrence et al., 1991b). Nevertheless in the periodate oxidation seems to be more confused the detection of GTX2,3 and STX because as it was mentioned these toxins co-eluted with the oxidation products of GTX1,4 and NEO. On the other hand, the run time in the post-column system leads 30 min for each injection, while only 15 min are necessary in the pre-column oxidation method. However not many injections should be programmed within the pre-column oxidation method because the oxidized samples are not stable (Lawrence et al., 2005), this is not a problem in the post-column oxidation method.

An important issue that concerns both methods is the time required for each analysis; it is the major problem that many authors have attempted to solve. A modification of the gradient elution perfected by Lawrence (Lawrence et al., 1995), was used in order to shorten analysis time. Some authors uses an autosampler programme to carry out the automated periodate and peroxide oxidation reactions (Vale and Sampayo, 2001). Other authors have proposed modifications of the isocratic elution of Oshima to shorten analysis time. In the first run they used binary gradient elution to resolve GTX1-5, dcGTX2-3, dcSTX, NEO and STX and in the second run they used an isocratic gradient to resolve C1-4 (Rourke et al., 2008; Jester et al., 2009). The time to elute the three groups of toxins remains lengthy and it is still an issue that should be further resolved. Ultra Performance Liquid Chromatography might be the solution of this problem.

As it was mentioned in the post-column oxidation method each toxin produces one peak. In this case, any sample can be correctly identified and quantified by using toxin standards, even though the available standard solutions are mixture of two toxins (GTX2,3, GTX1,4 and dcGTX2,3). However in the pre-column oxidation method these toxins are not separated and the amount of each one should be calculated according to the composition of these toxins in those solutions. Therefore if the sample has not the same profile than the standard the quantification can be wrong. It is necessary to bear in mind that the available standards are obtained after chemical conversion of toxins from dinoflagellates cultures and epimerized to an equilibrium ratio using pH6 and heat. This fact is highlight pointed in the present paper since the amount of each toxin obtained either by pre- or post- column oxidation methods is the same when samples from mussels are analyzed. However when the samples are from dinoflagellates and no chemical conversion is done the amount of each toxin calculated by both methods is different. As it can be observed the amount of GTX3 and GTX4 is higher than the amount of GTX2 and GTX1 in dinoflagellates cultures, while in the available standards these proportions are reversed. Therefore when two toxins produce only one peak (pre-column oxidation method) it is not accurate to use the standard percentage to know the amount of each toxin or the correct toxin profile of a sample.

In conclusion, the post-column HPLC method is the best option to identify and quantify each PSP toxin when non-transformed samples are analyzed, however when mussels samples are analyzed (or any transformed sample) the pre-column HPLC method is a useful technique.

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Conflict of interest

None declared.

References

Alfonso, A., Vieytes, M.R., Botana, A.M., Goenaga, X., Botana, L.M., 1993. Preparation of mixtures of paralytic shellfish toxin (PSP) standards from mussel hepatopancreas. Fresenius. J. Anal. Chem., 212–216.

AOAC, 2005. Paralytic Shellfish Poison. Biological Method. First Action 1959. Official Methods of Analysis of the AOAC. Method 49.10.01.

Ben-Gigirey, B., Rodríguez-Velasco, M.L., Villar-González, A., Botana, L.M., 2007. Influence of the sample toxic profile on the suitability of a high performance liquid chromatography method for official paralytic shellfish toxins control. J. Chromatogr. A., 78–87.

Ben-Gigirey, B., Villar-González, A., 2008. Chemical analysis. In: Botana, L. M. (Ed.), Seafood and Freshwater Toxins: Pharmacology, Physiology, and Detection. Taylor & Francis, Boca Raton-New York, pp. 177–193.

Chen, C.Y., Chou, H.N., 2002. A modified high-performance liquid chromatography method for analysis of PSP toxins in dinoflagellate, Alexandrium minutum, and shellfish from Taiwan. Food Res. Int. 35, 715–720.

Dell'Aversano, C., Walter, J.A., Burton, I.W., Stirling, D.J., Fattorusso, E., Quilliam, M.A., 2008. Isolation and structure elucidation of new and unusual saxitoxin analogues from Mussels. J. Nat. Prod. 71, 1518–1523.

E.U.C, 2006. Commission regulation (EC) No. 1664/2006 of 6 November 2006 amending regulation (EC) No 2074/2005. Official Journal of the European Union. Off. J. Eur. Union L 320, 13.

Franco, J.M., Fernández-Vila, P., 1993. Separation of Paralytic shellfish toxins by reversed phase high performance liquid chromatography, with postcolumn reaction and fluorimetric detection. Chromatographia 35, 613–620.

Gago-Martinez, A., Moscoso, S.A., Leao Martins, J.M., Rodriguez Vazquez, J. A., Niedzwiadek, B., Lawrence, J.F., 2001. Effect of pH on the oxidation of paralytic shellfish poisoning toxins for analysis by liquid chromatography. J. Chromatogr. A 905, 351–357.

Hallegraeff, G.M., 1995. Harmful Algal Blooms: a global overview. In: Hallegraeff, G.M., Anderson, D.M., Cembella, A.D. (Eds.), Manual on Harmful Marine Microalgae, IOC Manuals and Guides No. 33. UNESCO, pp. 1–22.

Jester, R., Rhodes, L., Beuzenberg, V., 2009. Uptake of paralytic shellfish poisoning and spirolide toxins by paddle crabs (*Ovalipes catharus*) via a bivalve vector. Harmful Algae 8, 369–376.

Kodama, M., 2000. In: Botana, L.M. (Ed.), Seafood and Freshwater Toxins: Pharmacology, Physiology and Detection. Marcel Dekker Inc., New York, pp. 125–149.

Lawrence, J.F., Menard, C., 1991a. Liquid chromatographic determination of paralytic shellfish poisons in shellfish after prechromatographic oxidation. J. Assoc. Off. Anal. Chem. 74, 1006–1012.

Lawrence, J.F., Menard, C., Charbonneau, C.F., Hall, S., 1991b. A study of ten toxins associated with paralytic shellfish poison using prechromatographic oxidation and liquid chromatography with fluorescence detection. J. Assoc. Off. Anal. Chem. 74, 404–409.

Lawrence, J.F., Menard, C., Cleroux, C., 1995. Evaluation of prechromatographic oxidation for liquid chromatographic determination of paralytic shellfish poisons in shellfish. J. AOAC. Int. 78, 514–520.

Lawrence, J.F., Niedzwiadek, B., 2001. Quantitative determination of paralytic shellfish poisoning toxins in shellfish by using prechromatographic oxidation and liquid chromatography with fluorescence detection. J. AOAC. Int. 84, 1099–1108.

Lawrence, J.F., Niedzwiadek, B., Menard, C., 2005. Quantitative determination of paralytic shellfish poisoning toxins in shellfish using prechromatographic oxidation and liquid chromatography with fluorescence detection: collaborative study. J. AOAC. Int. 88, 1714–1732.

Lawrence, J.F., Wong, B., Menard, C., 1996. Determination of decarbamoyl saxitoxin and its analogues in shellfish by prechromatographic oxidation and liquid chromatography with fluorescence detection. J. AOAC. Int. 79, 1111–1115.

Luckas, B., Hummert, C., Oshima, Y., 2004. Analytical methods for PSP. In: Hallegraeff, G.M., Anderson, D.M., Cembella, A.D. (Eds.), Manual on Harmful marine Microalgae. Unesco Publishing, France, p. 191.

Oshima, Y., 1995. Postcolumn derivatization liquid chromatographic method for paralytic shellfish toxins. J. AOAC. Int. 78, 528–532.

Rourke, W.A., Murphy, C.J., G, P., Van de Riet, J.M., Burns, B.G., Thomas, K. M., Quilliam, M.A., 2008. Rapid postcolumn methodology for determination of paralytic shellfish toxins in shellfish tissue. J. AOAC. Int. 91 (3), 589–597.

Sommer, H., Meyer, K.F., 1937. Paralytic shellfish poisoning. Arch. Pathol. 24, 560–598.

Thomas, K., Chung, S., Ku, J., Reeves, K., Quilliam, M.A., 2006. In: Henshilwood, K., Deegan, B., McMahon, T., Cusack, C., Keaveney, S., Silke, J., O'cinneide, M., Lyons, D., Hess, P. (Eds.), Molluscan Shellfish Safety. The Marine Institute, Galway, Ireland, pp. 132–138.

Vale, C., Alfonso, A., Vieytes, M.R., Romaris, X.M., Arevalo, F., Botana, A.M., Botana, L.M., 2008. In vitro and in vivo evaluation of paralytic shellfish poisoning toxin potency and the influence of the pH of extraction. Anal. Chem. 80, 1770–1776.

Vale, P., Sampayo, M.A.M., 2001. Determination of paralytic shellfish toxins in Portuguese shellfish by automated pre-column oxidation. Toxicon 39, 561–571.

Van Dolah, F.M., Ramsdell, J.S., 2001. Review and assessment of in vitro detection methods for algal toxins. J. AOAC. Int. 84, 1617–1625.

3.4. Sección III: Desarrollo de un sistema de monitorización y alerta temprana de floraciones tóxicas en las costas europeas (en el contexto de un proyecto europeo SPIES-DETOX).

Actualmente el síndrome PSP es uno de los más extendidos a nivel mundial, seguido por el DSP, incluyendo las YTX, AZAs y PTXs. Todas estas toxinas son un problema de vital importancia en las costas europeas. Se han detectado altos niveles de toxinas PSP, DSP y ASP que han llevado al cierre de zonas de recolección de moluscos en toda Europa durante muchos meses. Para minimizar las pérdidas económicas que esto supone y para asegurar la calidad de los productos, la industria necesita mejorar los sistemas de gestión de riesgos. De hecho, desde hace años funcionan programas de vigilancia para la detección de las microalgas en la columna de agua y para el estudio de los niveles de toxicidad en los moluscos. Sin embargo, es necesario implantar sistemas de monitorización más efectivos y constantes en el tiempo que permitan controlar la presencia de algas tóxicas en el agua del mar y las toxinas producidas y establecer así una relación entre ambas.

En el presente trabajo y en el contexto de un proyecto europeo (SPIES DETOX) [389] se estudió la adsorción pasiva de toxinas PSP y DSP disueltas en agua de mar sobre resinas sintéticas y/o polímeros y la posterior extracción y detección de estas toxinas. La idea es establecer un sistema sencillo y efectivo de alerta temprana de las floraciones de algas tóxicas que impida la contaminación de los moluscos. Además, este sistema permitirá proteger a los consumidores, facilitar la gestión comercial de las zonas de cultivo y evitar la recolección de producto contaminado.

A esta parte corresponden las siguientes publicaciones:

III.1: Study of solid phase adsorption of paralytic shellfish poisoning toxins (PSP) onto different resins.

III.2: Utilisation of solid-phase adsorption toxin tracking (SPATT) as a tool to monitor the presence of lipophilic shellfish toxins in the coastal waters of Western Europe.

III.1: Estudio de la adsorción en fase sólida de las toxinas causantes de la intoxicación paralítica por consumo de molusco (PSP) sobre diferentes resinas.

Resumen

El polímero diseñado computacionalmente (CDP), basado en el monómero funcional de fosfato de metacrilato de etilenglicol (EGMP) tiene una alta afinidad específica para toxinas neurotóxicas causantes de la intoxicación PSP como la STX y la neoSTX. Este polímero se evaluó con el fin de ser incluido en el sistema de seguimiento de la toxina en fase sólida (SPATT) para su implementación en el medio marino. Conjuntamente, se evaluó el poder adsorbente de una resina sintética (SEPABEADS® SP700), la cual había sido utilizada anteriormente en estudios de adsorción de toxinas lipofílicas y había resultado eficaz. Se investigó por lo tanto, la adsorción y eliminación de las toxinas PSP sobre y desde el polímero CDP y la resina SP700. Ambos adsorbentes se depositaron dentro de bolsas hechas con membrana de diálisis y estas a su vez se introdujeron en agua de mar contaminada con toxinas PSP y en cultivos de *Alexandrium tamarense*, productor de toxinas PSP. Además, algunas de las bolsas, conteniendo la resina SP700 también se introdujeron en una mezcla de cultivos de dinoflagelados compuesta por *Alexadrium tamarense* y *Prorocentrum lima*, siendo este último un productor de toxinas DSP. El polímero y la resina fueron extraídos y analizados para las toxinas PSP utilizando la técnica de HPLC. Así mismo, la resina SP700 también fue analizada para las toxinas DSP utilizando la técnica de LC-MS. Los resultados indicaron que tanto CDP como SP700 son adecuados para la adsorción de un amplio rango de toxinas PSP en un período limitado de tiempo (3-7 días). El polímero CDP parece ser más adecuado, ya que adsorbe mayores cantidades de toxinas PSP, sin embargo la resina SP700 adsorbe toxinas PSP y DSP en el mismo rango y por lo tanto puede ser más útil en programas de control. Las toxinas PSP se pueden eliminar fácilmente de ambos materiales con el agua de lavado o en un medio libre de toxinas. En resumen, tanto el polímero CDP como la resina SP700 podrían ser utilizados como un sistema de alerta temprana para el seguimiento de las HABs responsables de la intoxicación por PSP en aguas costeras europeas.

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Study of solid phase adsorption of paralytic shellfish poisoning toxins (PSP) onto different resins

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ABSTRACT

A computationally designed polymer (CDP), based on the functional monomer ethylene glycol methacrylate phosphate (EGMP), with a reported high specific affinity for the neurotoxic paralytic shellfish poisoning (PSP) toxins saxitoxin (STX) and neosaxitoxin (neoSTX) was evaluated with a view to it being used in a solid phase adsorption toxin tracking (SPATT) system for deployment in the marine environment. In addition, a synthetic resin adsorbent (SEPABEADS® SP700) which had previously shown to adsorb lipophilic shellfish toxins (LSTs) from seawater was also assessed. Adsorption and desorption of the PSP toxin analogues on and from the CDP polymer and the SP700 resin were investigated. Both adsorbents were contained within dialysis membrane bags and deployed in PSP toxins-spiked seawater and in cultures of *Alexandrium tamarense* known to be producing PSP toxins. Additionally, some bags holding SP700 resin were also deployed in a mixture of dinoflagellate cultures composed of *A. tamarense* and *Prorocentrum lima*, the latter being a diarrheic shellfish toxins (DSP) producer.

The polymer and the resin were extracted and analysed for PSP toxins using high performance liquid chromatography (HPLC). In addition, the SP700 resin was also analysed for DSP toxins using liquid chromatography–mass spectrometry (LC–MS).

The results indicated that both CDP and SP700 are suitable for the adsorption of a wide range of PSP toxins for a limited period of time (3–7 days). The CDP appears to be more appropriate because it adsorbs higher quantities of PSP toxins, however SP700 adsorbing PSP and DSP toxins over the same range seems to be more useful. PSP toxins can be easily removed from both materials with rinse water or in a toxin-free medium. In summary, either CDP or SP700 could be used as an early warming system for the monitoring of harmful algal blooms (HAB) responsible for PSP poisoning found in European coastal waters.

1. Introduction

The occurrence of harmful algal blooms (HAB) and subsequent contamination of shellfish with biotoxins produced by micro-algae is a recurrent public health problem. Because of this and the serious implications of economic loss to the shellfish fisheries and aquaculture industry, many countries have invested in regulatory systems including the routine monitoring of shellfish flesh for the presence of biotoxins and the examination of water samples for the presence of toxin producing phytoplankton (Hallegraeff, 1995).

The use of shellfish monitoring to assess the distribution of toxins in the marine environment remains the most appropriate method to ensure food safety even though shellfish sampling and analyses present several disadvantages. These can include difficulties in sample collection, handling and transport to the regulatory laboratories, specific species differences in toxin uptake and depuration, biotransformation of toxins in shellfish as well as analytical interferences and matrix effects (Ito and Tsukada, 2002; Stobo et al., 2005; Fux et al., 2008). In addition, laboratory analysis of shellfish is time consuming, technically demanding and expensive, so it is not ideal as a tool for monitoring the progression of toxigenic blooms (Rundberget et al., 2009).

Although phytoplankton monitoring has, in some cases, the ability to anticipate future shellfish toxin contamination, it is subjected to some serious limitations such as the high labour content by analysts trained in algal taxonomy (Rundberget et al., 2009). Besides, it only provides a snapshot of the algal population present at the time and location of the sampling and it is often difficult to establish a clear correlation between the presence of toxic phytoplankton and shellfish toxicity (Fux et al., 2009).

Recently, passive sampling techniques have been suggested as a tool to assist and improve marine biotoxin monitoring (MacKenzie et al., 2004; Vrana et al., 2005; Turrell et al., 2007; Fux et al., 2009; Rundberget et al., 2009). Prior to their recent application to the

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field of marine biotoxins, passive sampling methods have demonstrated their ability to adsorb a wide range of environmental contaminants (e.g., polycyclic hydrocarbons, polychlorinated biphenyls and other persistent organic pollutants) over a specific deployment period (Vrana et al., 2005).

The use of passive samplers for the detection of marine biotoxins in the water column was founded on observations during HAB events and toxic algae culture studies where significant amounts of polar and non-polar biotoxins dissolved in the seawater were observed (MacKenzie et al., 1998, 2003). Field experiments in New Zealand showed a lag period between the detection of dissolved toxins in the water column using passive samplers, phytoplankton peak cell densities and maximum concentrations of toxins in shellfish tissue (MacKenzie et al., 2004). It was suggested that passive samplers could have potential as an early warning system for the appearance of shellfish biotoxin contamination.

So far, passive samplers for marine biotoxins, referred to as solid phase adsorption toxin tracking (SPATT), have only been applied to the detection of lipophilic shellfish toxins (LSTs) in the water column (MacKenzie et al., 2004; Turrell et al., 2007; Fux et al., 2009; Rundberget et al., 2009). Another group of marine biotoxins, paralytic shellfish poisoning (PSP) toxins characterised by their ability to block voltage-gated sodium channels (Shimizu, 2000), are probably the most difficult to be sampled in natural waters because of their low concentrations and their relatively high hydrophilicity (Chan et al., 2005). Analytical protocols have been developed for the routine monitoring of shellfish samples where the concentrations of PSP toxins are relatively high due to bioaccumulation (Fang et al., 2004). Investigating the capacity of SPATT to accumulate dissolved PSP toxins from the water column could potentially serve as an early shellfish toxin contamination warning system for areas important to shellfish harvesting.

This paper describes the investigation carried out to assess the capacity of a computationally designed polymeric (CDP) material and a synthetic adsorbent SP700 resin to adsorb PSP toxins from spiked seawater and from cultures of PSP toxin producer *Alexandrium tamarense*. Additionally, the SP700 resin was also deployed in mixed cultures of *A. tamarense* and *Prorocentrum lima*, the latter being a DSP toxins producer, to investigate any variation of adsorption efficiency for the PSP toxins in the presence of okadaic acid (OA) and dinophysis toxin-1 (DTX-1) in the culture medium. Subsequent desorption, recovery and analysis of the toxins is also discussed.

2. Materials and methods

2.1. Reagents

HPLC grade methanol, acetonitrile, acetic acid, sodium hydroxide, periodic acid, disodium hydrogen phosphate, hydroxide peroxide and trifluoroacetic acid (TFA) were obtained from Panreac Quimica S.A. (Barcelona, Spain). Ammonium formate and formic acid were purchased from Sigma–Aldrich (Spain) and Merck (Spain) respectively. Ultra-pure water was obtained from an Arium 611 system (Sartorius, Germany) connected to a pre-purification filter system.

Standard solutions of saxitoxin (STX), neosaxitoxin (neoSTX), gonyautoxins 1 and 4 (GTX1, 4), gonyautoxins 2 and 3 (GTX2, 3), C1 and C2, GTX 5, DTX-1 and OA were purchased from the Certified Reference Material Program of the National Research Council of Canada (Institute for Marine Biosciences, Halifax, Canada) and CIFGA S.A. (Lugo, Spain).

The CDP with a particle size of 38–106 μm was prepared from the functional monomer ethylene glycol methacrylate phosphate (EGMP) by Prof. S. Piletsky (Cranfield University, United Kingdom).

The synthetic adsorbent resin SEPABEADS® SP700 (PS-DVB adsorbent) was obtained from Mitsubishi Chemical Corporation.

Spiked seawater was prepared by adding different amounts of PSP toxins purified from contaminated mussels. These toxins were obtained after whole flesh extraction (ethanol/acetic acid) followed by purification through exclusion chromatography (Alfonso et al., 1993). The purity of toxin extracts was always higher than 90%.

2.2. Cell cultures

The strain of *A. tamarense* (04/197/A1) used in the experiments was isolated from Scottish coastal waters. The strain of *P. lima* (CCMP 686) was provided by the Bigelow Laboratory for Ocean Sciences (ME, USA). Both strains were maintained in L1 culture medium on 14:10 L/D cycles at 19 ± 1 °C. Both cultures were used after exponential phase.

2.3. CDP samplers

A dialysis membrane with a molecular weight cut-off (MWCO) of 12.4 K was used to prepare bags of approximately 3 cm × 6 cm in size. The bags were filled with 100 mg of CDP previously conditioned with 2 mL of HPLC-grade water for 10 min. The bags with the CDP were then closed with dialysis closures. Several experiments were carried out to test PSP adsorption by the CDP.

Initially, the dialysis bags filled with CDP were placed in 5 mL tubes containing seawater spiked with PSP toxins (0.3 μg/mL of STX, 0.5 μg/mL of GTX2, 3 and 2.5 μg/mL of C toxins) obtained from purified mussel extracts. The bags were gently shaken and incubated at 8 and 19 °C for a period of one week. Aliquots of 200 μL seawater were taken every day and PSP toxins were quantitatively analysed. After 7 days, the bags were removed from the tubes and the PSP toxins were extracted.

In another set of experiments, the *A. tamarense* cultures into Erlenmeyer flasks (200 mL) were prepared. The cells were firstly grown in controlled conditions until post-exponential phase was reached with densities around 5000 cells/mL. Then, the CDP dialysis bags attached to a glass weight to avoid floatation were deployed in the culture mediums and removed from these at 1 h, 1, 2, 3 and 7 days. In addition, an Erlenmeyer flask (200 mL) containing only *A. tamarense* culture (without bags) was used as control. These experiments were carried out in an incubator with the following settings: temperature 19 ± 1 °C, light–dark cycles (14 h–10 h), L1 medium. Media aliquots were removed daily from all vessels to count the number of cells and to follow culture viability.

After 7 days, the cultures were filtered by gravity through a 10 μm nylon mesh. The filtrated cells were suspended in acetic acid/ethanol (3:1), with a ratio of three volumes of solvent per cell volume and then broken by ultrasound (three cycles of 20 s) while cooling in ice bath. Then, the extracts were centrifuged at 3000 rpm for 10 min at 4 °C. The supernatants were separated from the pellets, evaporated to dryness and finally dissolved in 500 μL acetic acid (0.03 N) before being analysed for PSP toxins by HPLC.

Other experiments were performed to asses if desorption of PSP toxins from the CDP was possible when the resin is deployed into seawater toxins-free. For this, four bags filled with CDP were deployed in *A. tamarense* cultures (200 mL). Two of the bags were collected from the culture medium after three days: the resin contained in one bag was extracted and the other bag was re-deployed in a vessel containing seawater without toxins for four additional days before final extraction of the resin. The other two bags which had been left incubating in the *A. tamarense* culture for seven days were removed: the resin contained in one bag was

immediately extracted while the other bag was moved into a vessel filled with toxin-free seawater and was left incubating for four days before final extraction of the resin.

2.4. CDP extraction and regeneration

After incubation, each CDP dialysis bag was opened and the resin (100 mg) was transferred to a 2 mL column from Supelco (Bellefonte, USA) installed on a Chromabond® vacuum manifold from Macherey-Nagel (Düren, Germany). The resin was then immediately rinsed with 6 mL of ultra-pure water and the PSP toxins were eluted twice with a solution of 10% methanol (3.5 mL) containing 2% trifluoroacetic (TFA). Finally, the rinsed water fraction and the two methanolic fractions were vacuum dried in a miVac centrifugal concentrator from Genevac (Ipswich, UK). The residues were dissolved in 500 μL acetic acid (0.03 N) and analysed by HPLC using the Lawrence method (Lawrence et al., 2005) to quantify the presence of any PSP toxins.

After extraction, the CDP resin was regenerated following consecutive washings: ultrapure water (10 mL) first followed by 2% TFA in methanol (10 mL) and finally ultrapure water (10 mL). The CDP was reused throughout the experimentation due to high cost and limited quantities.

2.5. SP700 samplers

A dialysis membrane with a MWCO of 12.4 K was used to prepare bags of approximately 3 cm × 10 cm in size. The bags were filled with SP700 resin (1 g) previously conditioned with HPLC-grade water, closed with dialysis closures and then attached to a glass weight to avoid floatation in the medium.

The SP700 resin has previously been reported to be suitable for the adsorption of LSTs from seawater (Turrell et al., 2007). In the reported work, several experiments were performed to investigate the ability of the SP700 resin to adsorb PSP toxins.

Initially the SP700 dialysis bags were deployed in *A. tamarense* cultures for 3 and 7 days. The experiments were carried out following the culture and incubation conditions cited before in the CDP experiments. The SP700 bags were removed from the culture medium after 3 and 7 days and the resins extracted.

In another set of experiments, a mixture of PSP and LSTs producing cultures (*A. tamarense* and *P. lima*) was introduced in a Erlenmeyer flask (700 mL). The cells were grown in controlled conditions until post-exponential phase was reached. The SP700 dialysis bags were then added to the mixture of cultures and removed from these after 3 and 7 days respectively. These experiments were carried out in an incubator with the following settings: temperature 19 ± 1 °C, light–dark cycles (14 h–10 h), L1 medium. In addition, an Erlenmeyer flask containing only the mixture of cultures was used as control. Media aliquots were removed daily for seven days from all vessels to count the number of cells and to follow culture viability. After seven days, the cultures were filtered by gravity through a 10 μm nylon filter mesh, as described in Section 2.3.

As in CDP experiments, the toxin desorption from the SP700 resin was studied. Similarly to what was described before (Section 2.3), four SP700 filled bags were deployed in mixed *A. tamarense* and *P. lima* cultures for 3 and 7 days. Two bags were collected from the culture medium after 3 days. One of the bags was extracted while the second bag was deployed in an Erlenmeyer flask containing seawater for four days. The other two bags were removed from the mixed cultures medium after seven days. One of the bags was immediately extracted and the second bag was transferred into a vessel filled with toxin-free seawater and left incubating for four days.

2.6. SP700 extraction

After incubation, each SP700 dialysis bag was opened and the resin was transferred into a 25 mL cartridge fitted with a 20 μm frit from Ingenieria analitica S.L. (Barcelona, Spain) installed on a vacuum manifold. The resin was immediately rinsed with ultra-pure water (40 mL) then was soaked in methanol (2 mL) for 5 min. The cartridge and its contents were mixed (10 s), and then the methanolic fraction was collected. The resin was further eluted with 18 mL methanol and the eluent was pooled with the first fraction. An aliquot (10 mL) was taken from the water and methanolic fractions which then were vacuum dried, dissolved in 500 μL acetic acid (0.03 N) prior to PSP analysis by HPLC using the Lawrence method (Lawrence et al., 2005). Another aliquot (10 mL) of each fraction was taken out, vacuum dried, dissolved in 500 μL methanol then filtered through 0.45 μm Ultrafree-MC centrifugal filters from Millipore (Spain) prior to DSP analysis by LC–MS according to a previously detailed protocol (Alfonso et al., 2008).

2.7. PSP detection: HPLC

PSP toxins were analysed by HPLC with fluorescence detection after pre-chromatographic oxidation, the samples were oxidized using hydrogen peroxide to detect GTX2 and GTX3 (combined), GTX5, C1 and C2 (combined) and STX and periodate to detect GTX1 and GTX4 (combined) and NEO (Lawrence et al., 2005). A Supelcosil™ LC-18 (5 μm, 150 mm × 4.6 mm) column (Sigma–Aldrich, Spain) was used to separate the toxins. The column oven was at 35 °C. Two mobile phases were used. Mobile phase A was a solution of ammonium formate (0.1 M) acidified to pH 6 with acetic acid (1 M). Mobile phase B was a solution of ammonium formate (0.1 M) with 5% acetonitrile and acidified to pH 6 with acetic acid (1 M). Elution of the PSP oxidation products was carried out using the following gradient: 0–5% B for the first 5 min, 5–70% B for the next 4 min, back to 0% B over the next 2 min; then 0% B for 3 min before the next injection. The gradient was delivered by a Waters 515 HPLC binary pump. Injection volumes were 25 μL for samples oxidized with hydrogen peroxide and 100 μL for samples oxidized with periodate. The flow rate was set at 1 mL min^{-1} (Ben-Gigirey et al., 2007). The oxidized PSP toxins were detected by a Waters 2475 fluorescence detector, with excitation and emission set at 340 nm and 395 nm respectively. The HPLC system was controlled by the chromatography data software Empower™ (Waters Corporation, Spain). Quantitation of the PSP toxins in the sample extracts was determined by comparison of the peaks areas of the PSP toxins in the samples with those in calibrated standards.

2.8. DSP detection: LC–MS

The HPLC system consisted of two pumps (LC-10ADvp), an autosampler (SIL-10ADvp) with refrigerated rack, a degasser (DGU-14A), a column oven (CTO-10ACvp) and a system controller (SCL-10Avp) from Shimadzu (Japan). This system was coupled to a 2000 QTRAPLC/MS/MS system from Applied Biosystems (Bedford, MA, USA), consisting of an hybrid quadrupole-linear ion trap mass spectrometer (MS) equipped with atmospheric pressured ionisation (API) fitted with an electrospray ionisation source (ESI). Nitrogen was produced by a Nitrocraft $NC_{LC/MS}$ generator from Air Liquide (Spain). Separation of the DSP toxins was achieved using a BDS-Hypersil C8 3 μm 120 Å (50 mm × 2 mm) column from Phenomenex (USA) with a C8 guard cartridge (10 mm × 2.1 mm) mm) from Thermo (USA). Column oven temperature was set at 25 °C and injection volume was 5 μl. Mobile phase A consisted of a solution of ammonium formate (2 mM) and formic acid (50 mM) whereas the organic phase B consisted of acetonitrile:water (95:5) with ammonium formate (2 mM) and formic acid (50 mM).

Analyses were carried out using a linear gradient elution with a constant total flow of 0.2 mL min^{-1} and an analysis run time of 14 min. The gradient started at 30% B and reached 90% B after 8 min; was held at 90% B for 3 min; decreased to 30% B in 0.5 min and was maintained at 30% B for 2.5 min until the next analysis. Analyst software was used to control the system during analysis and process the data. Sample extracts were analysed with the ESI interface operating in negative ion mode under the following parameters: curtain gas 15; CAD gas 6; IonSpray voltage 4000 V; temperature 450 °C; gas 1 50 and gas 2 50; these parameters had previously been optimised using toxin standards (Alfonso et al., 2008). The mass spectrometer was operated in multiple reaction monitoring (MRM), monitoring two ion transitions per toxin: one for quantification and the other for confirmation. The transitions selected were 803.6 > 255.2/113.5 and 817.6 > 255.2/113.5 for OA and DTX-1 respectively. The most abundant ion in the fragment spectra at m/z 255.2 was used to quantify both OA and DTX-1.

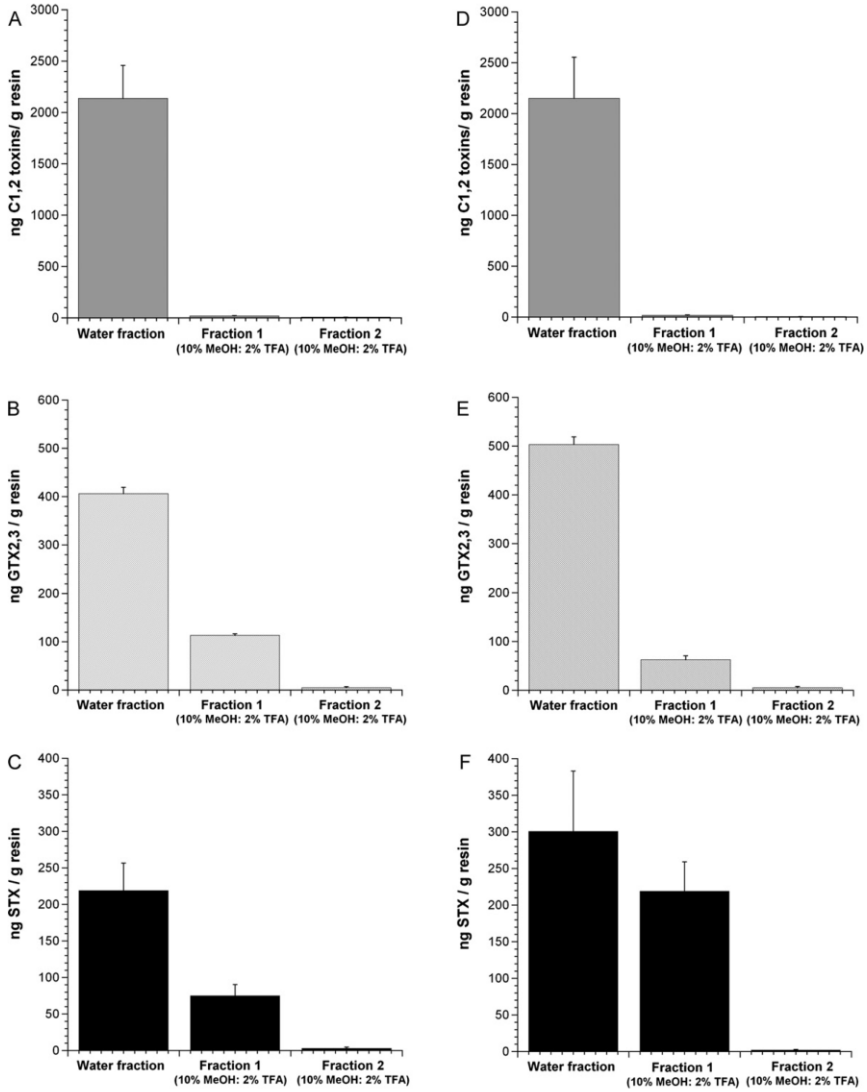

Fig. 1. PSP toxins concentrations (ng toxin/g resin) adsorbed onto CDP. After incubation at 8 and 19 °C the toxins were extracted with water followed by aqueous methanol with TFA (88:10:2, v/v/v). Concentrations of C1, 2 toxins (A), GTX2, 3 (B) and STX (C) in the water fraction and in the two acidified-methanolic fractions after incubation at 8 °C. Concentrations of C toxins (D), GTX2, 3 (E) and STX (F) in the water fraction and in the two acidified methanolic fractions after incubation at 19 °C. Mean ± SEM of three experiments.

3. Results

A series of experiments were carried out to investigate the adsorption and subsequent recovery efficiency of a range of PSP toxins onto CDP and SP700 resins. Initially, the bags filled with CDP were deployed in seawater spiked with PSP toxins. After 7 days exposure at 8 °C, the bags were removed from the medium and the toxins extracted and quantified. Fig. 1A–C shows the PSP toxins adsorption results after 7 days exposure at 8 °C. High concentration of C toxins (2136.98 ± 321.43 ng/g resin) were recovered from the CDP in the deionised water fraction while only 2% of the total concentration of C toxins were recovered in the acidified methanolic fractions (Fig. 1A). Similarly, GTX2, 3 and STX were also mainly recovered in the water fraction (406.02 ± 13.30 ng/g resin and 219.02 ± 37.71 ng/g resin respectively). However, a higher percentage of GTX2, 3 and STX, 23% and 26% respectively, were recovered in the organic fractions (Fig. 1B and C) compared to the C toxins. In order to investigate any potential temperature effect on the resin capacity to adsorb PSP toxins, the same experiment was repeated at 19 °C (Fig. 1D–F). The concentration of toxins recovered in the water fraction was 2152.35 ± 403.27 ng of Cs, 503.43 ± 15.43 ng of GTXs and 300.58 ± 82.56 ng of STX per g of resin. Under these conditions, the percentage of toxin recovered in the overall acidified methanolic fractions was 2% for the C toxins, 12% for GTX2, 3 and 40% for STX. These results show that a similar concentration of C toxins was recovered both at 8 and 19 °C while overall higher concentrations of GTX2, 3 and STX were recovered at 19 °C. These experiments showed the CDP resin was able to adsorb to a certain extent the hydrophilic PSP toxins. Toxin recoveries between 17% for the Cs toxins, 20% for GTX2, 3 and STX at 8° C were observed, whereas the recovery increased to 35% for STX at 19° C.

Similar experiments were then repeated with *A. tamarense* cultures to determine if the polymeric resin could adsorb the toxins produced by PSP toxin producing phytoplankton. The PSP toxin profile of the *A. tamarense* strain under the laboratory culture conditions is showed in Fig. 2A. The culture produced high concentrations of GTX1, 4, C1, 2 and neoSTX and low concentrations of STX and GTX2, 3. The number of cells was relatively constant during the experiment in both the control culture and in the culture with the CDP filled bags (Fig. 2B). The amount of toxins released from the *A. tamarense* cells into the culture medium was also checked during the 7 days experimental period and was found to be constant (Fig. 2C). In the same way, bags filled with CDP were deployed in *A. tamarense* cultures and time-removed (Section 2.3). The overall final concentration of each respective toxin extracted was calculated by adding the toxins recovered from the water fraction and the toxins recovered from the two acidified methanolic fractions. The adsorption of the toxins onto the resins increased from the first sampling point (1 h) until sampling at day 7 (Fig. 3). While C1, 2 and GTX1, 4 were detected in higher amount in the resin retrieved from the medium after three days with concentrations of 173 and 427 ng/g, respectively; GTX2, 3, STX and neoSTX were detected in higher amount in the resin deployed for seven days with concentrations of 60, 81 and 176 ng/g, respectively. In general, similar toxin amounts were detected both in the resin deployed for 3 days as in the resin deployed for 7 days suggesting that the CDP reached a maximum toxin adsorption between day 3 and day 7.

Then, the toxin desorption from the resin to toxin-free seawater was checked. Fig. 4 shows the results of the desorption experiments carried by soaking resin field bags in seawater for 4 days after the initial adsorption period in the *A. tamarense* culture. PSP toxins were desorbed from the CDP after four days (Fig. 4A). Desorptions of 80% for C1, 2, 68% for GTX1, 4, 67% for GTX2, 3, 60% for NEO, and 53% for STX were measured. Similar quantities of toxins desorbed were observed when the resin was

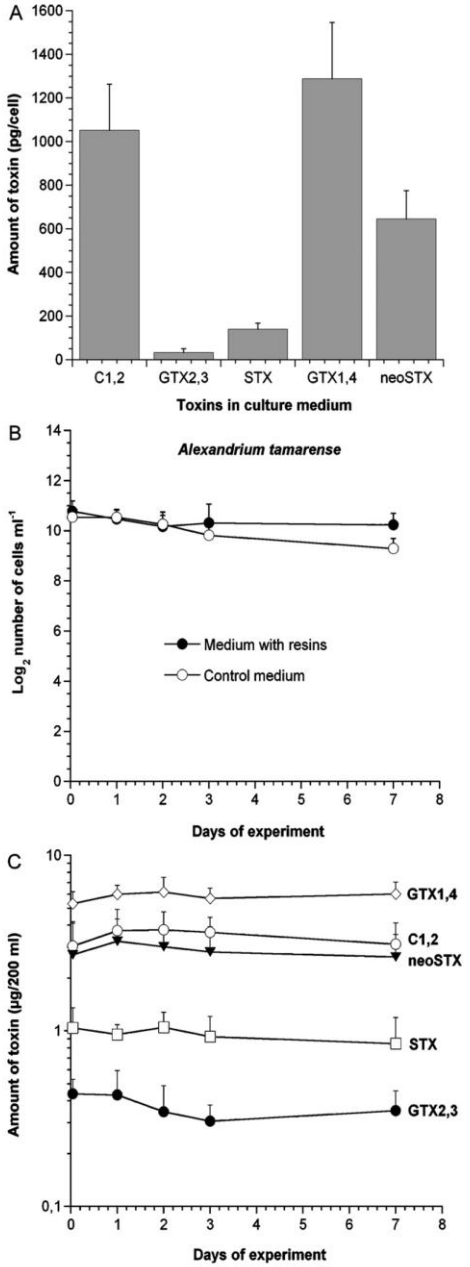

Fig. 2. Stability and viability of *A. tamarense* culture. (A) PSP toxin profile (pg/cell) of *A. tamarense* culture. (B) Evolution of the number of cells in the culture medium with and without resin bags. (C) Toxin concentration released from cells to the medium at experimental conditions. Mean ± SEM of three experiments.

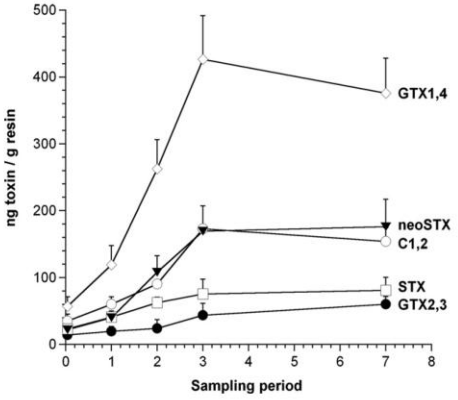

Fig. 3. Final concentration of PSP toxins (ng toxin/g resin) extracted from CDP resin after 1 h, 1, 2, 3, and 7 days incubation in *A. tamarense* culture. Mean ± SEM of three experiments.

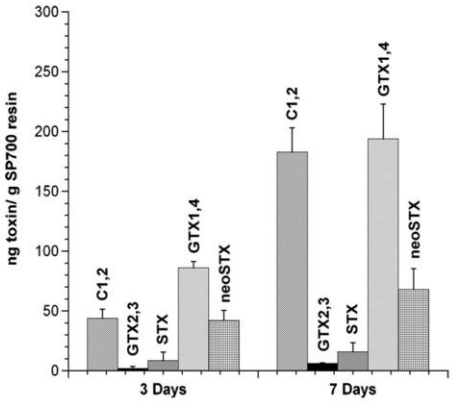

Fig. 5. Total amount of PSP toxins (ng toxin/g SP700 resin) recovered from SP700 filled bags after 3 and 7 days incubation in *A. tamarense* culture. Mean ± SEM of three experiments.

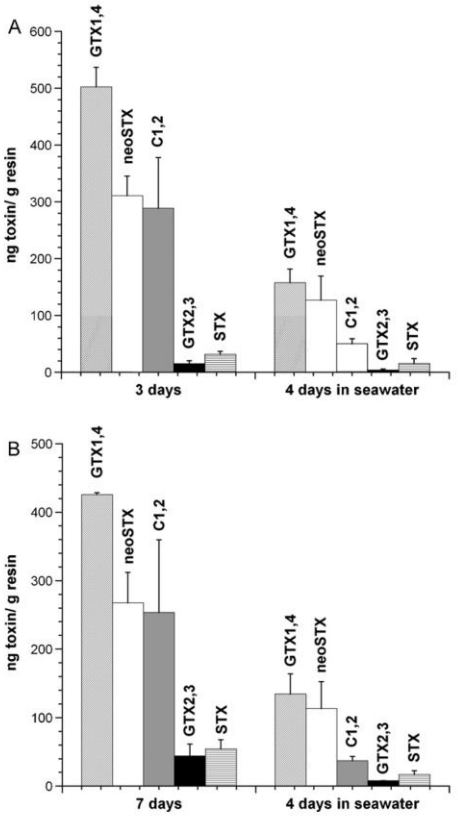

Fig. 4. PSP toxin concentrations (ng toxin/g resin) extracted from the CDP resin after 3 (A) and 7 (B) days incubation in *A. tamarense* cultures and after 4 subsequent days in toxin-free seawater. Mean ± SEM of three experiments.

firstly deployed for seven days in the *A. tamarense* culture and subsequently incubated for four days in seawater (Fig. 4B): loss of 81% for C1, 2, 79% for GTX2, 3, 69% for STX, 68% for GTX1, 4 and 59% for NEO respectively.

Although high amounts of toxins were desorbed from the CDP after 4 days incubation in toxin-free seawater, the quantities of toxins still adsorbed onto the resin are high enough to be detectable.

A new series of experiments were carried out to assess adsorption and recovery of PSP toxins on the SP700 resin. Initially, the SP700 filled bags were deployed in *A. tamarense* cultures. The bags were removed from the medium after 3 and 7 days and the resins extracted. The total amount of toxins extracted from the SP700 resin was much higher after seven days incubation in the culture medium (Fig. 5): 194 ng of GTX1, 4, 183 ng of C1, 2, 68 ng of neoSTX, 16 ng of STX and 6 ng of GTX2, 3 per gram of resin. These data demonstrate the SP700 resin has the ability to also adsorb PSP toxins.

In subsequent experiments, SP700 filled bags were deployed in mixtures of *A. tamarense* and *P. lima* cultures. Cell survival, toxin production and culture conditions of the mixture were firstly assessed. As showed, Not only C1, 2, GTX1, 4, Neo, STX and GTX2, 3 but also DTX-1 and OA are present in the mixed cultures medium (Fig. 6A). Similarly to the CDP filled bags, the presence of SP700 filled bags did not affect cell survival and the number of cells during the experiment was constant (Fig. 6B). In addition, the toxin released into the medium was also constant (Fig. 6C). After 3 and 7 days, the bags were removed from the medium and the toxins extracted. PSP toxins as well as OA and DTX-1 were recovered from the SP700 resin (Fig. 7A and B). The PSP toxins were mostly recovery in the water fraction while the OA and DTX-1 were mainly recovered in the methanol fraction. The quantity of toxins recovered is higher after seven days, especially the amount of OA and DTX-1 in the methanolic fractions (Fig. 7B).

Since high quantities of PSP toxins were desorbed from the CDP after resin incubation in toxin-free seawater, desorption from the SP700 resin was also studied (Fig. 8). After 3 days in the culture medium and 4 days in toxin-free seawater, Fig. 8A, the percentage of toxin desorbed was high for the PSP toxins (95% for C1, 2, 84% for GTX1, 4, 77% for neoSTX, 72% for GTX2, 3, 57% for STX) and much lower for the LSTs toxins (26% for OA and 4% for DTX1). After 7 days in the culture medium followed by 4 days in seawater (Fig. 8B),

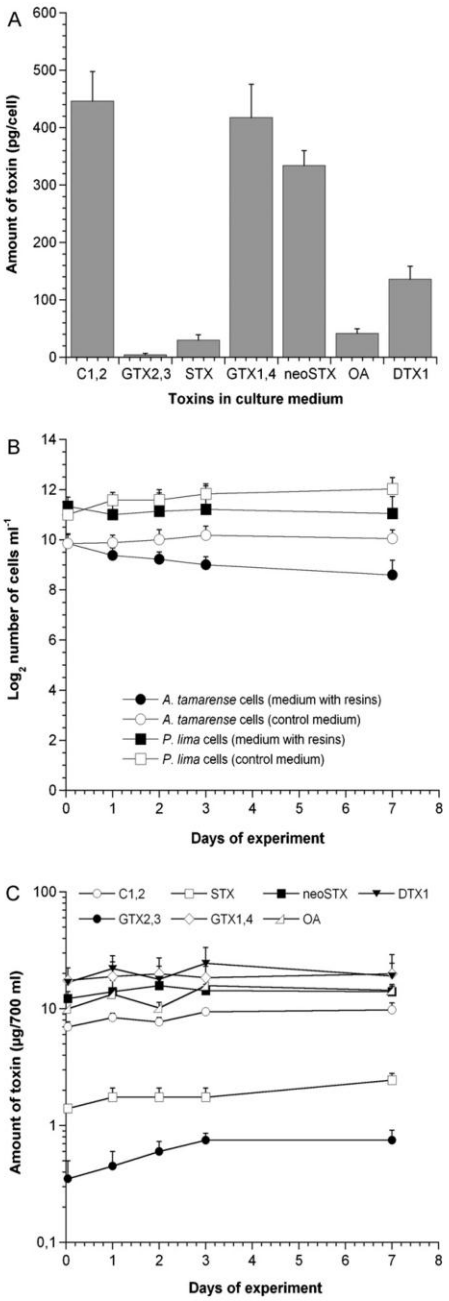

Fig. 6. Stability and viability of the mixed *A. tamarense* and *P. lima* culture. (A) PSP and DSP toxin profile in the mixed culture (pg/cell). (B) Number of *A. tamarense* and *P. lima* cells in the culture media with and without resin. (C) Evolution of the toxins' concentrations released from the *A. tamarense* and *P. lima* culture cells into the medium at experimental conditions. Mean ± SEM of three experiments.

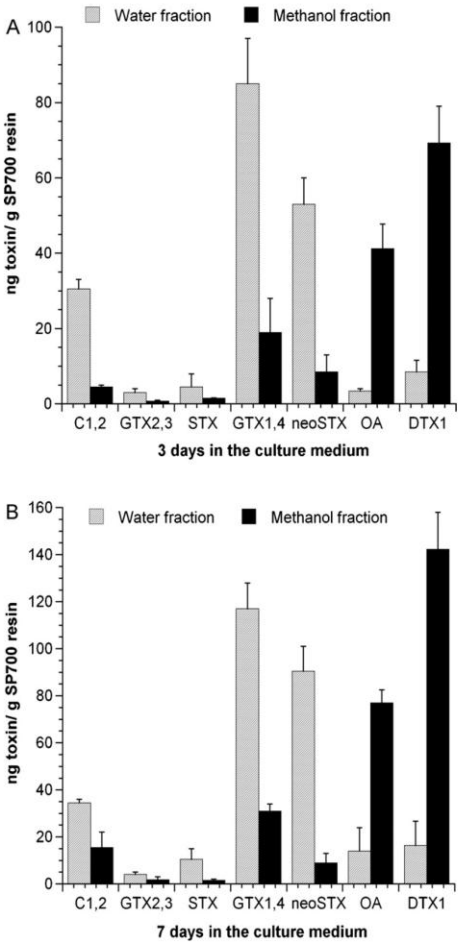

Fig. 7. Concentrations of toxins (ng/g of resin) in extracted SP700 resin bags after 3 and 7 days incubation in a mixture of *A. tamarense* and *P. lima* cultures. (A) Concentrations of toxins in water and methanol fractions after 3 days incubation in culture medium. (B) Concentrations of toxins in water and methanol fractions after 7 days incubation in culture medium. Mean ± SEM of three experiments.

similar toxin desorbed percentages were observed: high losses for PSP toxins (C1, 2 91%, STX 83%, GTX2, 3 82%, neoSTX 80%, GTX1, 4 78%) and lower losses for LSTs toxins (OA 35% and DTX1 23%).

In summary, adsorption of PSP toxins to CDP and SP700 resins was effective when incubated in dinoflagellates toxin producing cultures after short exposure periods. However high percentages of PSP toxins were desorbed from these resins after several days incubation in toxin-free seawater.

4. Discussion

PSP toxins such as STX, neoSTX and the GTXs constitute the most dangerous group of toxins produced by dinoflagellates blooms in coastal waters throughout the world. Routine monitoring to check for their presence is therefore mandatory in shellfish harvesting areas. The results described in this paper demonstrate

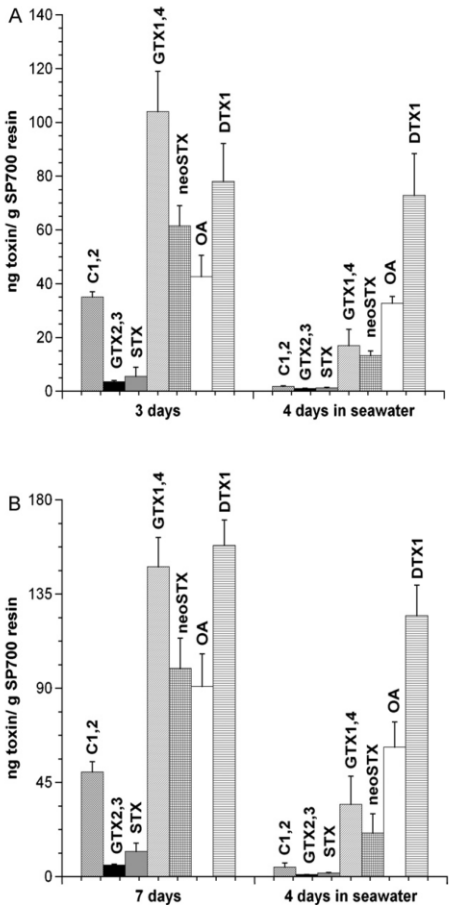

Fig. 8. PSP toxin concentrations (ng/g resin) recovered from SP700 filled bags. (A) Concentrations of toxins extracted from SP700 resin after 3 days incubation in a mixture of *A. tamarense* and *P. lima* cultures and after 4 days subsequent incubation in toxin-free seawater. (B) Concentrations of toxins extracted from SP700 resin after 7 days incubation in the same culture mixture as in A and after 4 days incubation in toxin-free seawater. Mean ± SEM of three experiments.

that the CDP resin is suitable for the adsorption of a wide range of PSP toxins. In addition, the SP700 resin which showed its ability to adsorb LSTs toxins, appeared to be also capable to adsorb PSP toxins.

The use of different resins (i.e. SP700, HP20) for toxins adsorption has been tested and validated by other teams (MacKenzie et al., 2004; Turrell et al., 2007; Fux et al., 2009; Rundberget et al., 2009). However, these resins have only been applied to the detection of algal blooms events which resulted in the release in the water column of polyether biotoxins (OA and dinophysistoxins, pectenotoxin-2, yessotoxin, azaspiracids). There is therefore a need to investigate resin adsorption using similar methods with specificity towards PSP toxins. In recent years, CDPs have become more frequently used in sorbent material for solid phase extraction (SPE) to cleanup complex matrixes of unwanted interfering small molecules (Chianella et al., 2002; Chapuis et al., 2004). More specifically, the design of such polymers using different functional monomers capable of binding PSP toxin (STX and neoSTX) was investigated (Turrell et al., 2008). After screening a selection of candidate monomers, three molecules were identified as being possibly suitable monomers to produce CDP material used in SPE for the adsorption of PSP toxins (Turrell et al., 2008). The three monomers were: 2-(Trifluoromethyl) acrylic acid (TFMAA), itaconic acid (IA) and ethylene glycol methacrylate phosphate (EGMP). It was thought these polymers could be useful molecules for the solid phase adsorption of PSP toxins dissolved in seawater. The EGMP based CDP was used in this work due to the reported strong binding between the sulfonic groups of the PSP toxins and the phosphate groups of the CDP (Turrell et al., 2008). In this study, the adsorption of PSP toxins onto the SP700 resin was also investigated. This resin has been used in laboratory trials and field deployments (SPATT bags) in Scotland for the monitoring of LSTs dissolved in seawater (Turrell et al., 2007).

In our experiments, the SP700 resin was deployed in mixed cultures of *A. tamarense* and *P. lima* in order to assess the adsorption capacity of PSP in the presence of DSP toxins (OA and DTX1). The results showed that the adsorption of both groups of toxins onto SP700 was around 1%. When the CDP filled bags were deployed in *A. tamarense cultures*, the adsorption of the PSP onto the polymer ranged was between 4 and 20%. Therefore CDP appears to be more suitable for the adsorption of PSP toxins than SP700, even at low toxin concentrations. The CDP could be an interesting candidate to be used in SPATT deployments to track PSP toxins in the water column.

A disadvantage of both CDP and SP700 resins is the fast toxin desorption from the resins when these are deployed in toxin-free seawater, probably due to the high aqueous solubility of these compounds. The C toxins were approximately 80% desorbed, the GTXs 70%, while neoSTX and STX were around 60% desorbed. This shows that neoSTX and STX present a stronger adsorption capacity than the other PSP toxins to this particular CDP polymer. The loss of PSP toxins through desorption was similar with the SP700 resin while incubated in toxin-free seawater, whereas the OA and DTX-1 desorption was limited to 30% and 13% respectively. SP700 was shown in our experiments to successfully adsorb LSTs from seawater. In addition, the amount of PSP and DSP toxins adsorbed by SP700 in relation with the amount of toxins released to the medium is similar therefore this resin is also suitable for PSP adsorption. On the other hand, since PSP toxins desorption occurs with both types of resins, we think the CDP is better than the SP700 resin to adsorb higher PSP concentrations. However SP700 is much cheaper and has the ability to adsorb both PSP and lipophilic toxins.

Toxin extraction from the resins was an easy, fast and economical procedure. The PSP toxins are mainly desorbed from the CDP not only with deionised water but also with an acidified methanolic solvent (10% methanol with 2% TFA) necessary to extract the PSP toxins (STX and neoSTX) more strongly attached to the polymer. In the case of the SP700 resin, the PSP are mostly extracted with water, while the LSTs are desorbed with 100% methanol as previously described (Turrell et al., 2007). It is important to point out that the PSP toxins are recovered from the CDP and the SP700 resins in the rinse water. It is therefore essential to consider the rinse water as a fraction in any extraction protocol in order to avoid the loss of toxins. This could be the reason why SP700 was not previously reported to be able to adsorb PSP toxins.

Additionally, both CDP and SP700 resin extracts have the advantage of being relatively matrix-free comparing to shellfish extracts. Endogenous compounds are extracted from the shellfish tissues along with the toxins which sometimes make the use of a

SPE cleanup step before analysis necessary. Overall, the elimination of matrix interferences from shellfish extracts before analysis decreases sample analysis turnover while increasing sample cost (Ben-Gigirey et al., 2007; MacKenzie, 2010).

Due to the rapid adsorption of toxins onto the resins which was observed to be, between 3 and 7 days, the ability for the resins to be an early warning tool may be possible if the transportation duration of toxic algae along coastal currents and tides is significantly longer than the duration of toxin adsorption on the resins. In the case of phytoplankton monitoring for example, very high levels of potentially toxic phytoplankton species were observed during weekly monitoring sampling, while monthly sampling was likely to miss their presence (Bresnan et al., 2005). The periodicity and duration of monitoring is very important. The deployment of the resin filled bags should not last longer than 7 days in the water to avoid toxin loss due to desorption as shown by our results.

The system of CDP/SP700 resin filled bags as passive samplers combined with sensitive detection methods such as HPLC and LC–MS, could provide detailed time-averaged information about the profile of hydrophilic toxins present in the water column. It is necessary to bear in mind that the amount of toxin adsorbed to the resin can be low; therefore a sensitive toxin detection method has a key role in the whole toxin detection and identification process. Possible detection of low concentrations of hydrophilic toxins produced at the beginning of a bloom of toxic *Alexandrium sp.* following resin adsorption may have the potential to provide a useful early warning tool. In contrast to conventional phytoplankton monitoring methods which might not be able to distinguish between toxic and non toxic species using solely light microscopy, resin filled bags directly targeting the toxic compounds of interest provide a serious advantage as a toxin monitoring method (MacKenzie, 2010).

Additionally, the resin approach could also be a useful tool to be implemented as a monitoring system in replacement of shellfish testing in the case of high selling value tested shellfish such as queen clams and oysters. The resin could therefore potentially replace the sentinel system based on caged mussels that sometimes are used to avoid wasting precious commercial products. However, before any implementation, further resin deployments during toxic events should be carried out to study in detail the relationship between shellfish toxic contamination levels and phytoplankton and toxins concentrations in the water column.

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References

Alfonso, A., Vieytes, M.R., Botana, A.M., Goenaga, X., Botana, L.M., 1993. Preparation of mixtures of paralytic shellfish toxin (PSP) standards from mussel hepatopancreas. Fresen. J. Anal. Chem. 345, 212–216.

Alfonso, C., Alfonso, A., Otero, P., Rodríguez, P., Vieytes, M.R., Elliot, C., Higgins, C., Botana, L.M., 2008. Purification of five azaspiracids from mussel samples contaminated with DSP toxins and azaspiracids. J.Chromatogr. B 865, 133–140.

Bresnan, E., Fryer, R., Hart, M., Percy, L., 2005. Correlation between algal presence in water and toxin presence in shellfish. Food Standards Agency UK Project Code B04005.

Ben-Gigirey, B., Rodríguez-Velasco, M.L., Villar-González, A., Botana, L.M., 2007. Influence of the sample toxic profile on the suitability of a high performance liquid chromatography method for official paralytic shellfish toxins control. J. Chromatogr. A 1140, 78–87.

Chan, I.O.M., Lam, P.K.S., Cheung, R.H.Y., Lam, M.H.W., Wu, R.S.S., 2005. Application of solid phase microextraction in the determination of paralytic shellfish poisoning toxins. R. Soc. Chem. 130, 1524–1529.

Chapuis, F., Pichon, V., Hennion, M.C., 2004. Molecularly imprinted polymers: developments and applications of new selective solid-phase extraction materials. LC GC Europe 17 (7), 408–417.

Chianella, I., Lotierzo, M., Piletsky, S.A., Tothill, I.E., Chen, B., Karim, K., Turner, A.P.F., 2002. Rational design of a polymer specific for microcystin-LR using a computational approach. Anal. Chem. 74, 1288–1293.

Fang, X., Fan, X., Tang, Y., Chen, J., Lu, J., 2004. Liquid chromatography/quadrupole time-of-flight mass spectrometry for determination of saxitoxin and decarbamoylsaxitoxin in shellfish. J. Chromatogr. A 1036 (2), 233–237.

Fux, E., Rode, D., Bire, R., Hess, P., 2008. Approaches to the evaluation of matrix effects in the liquid chromatography–mass spectrometry (LC–MS) analysis of three regulated lipophilic toxin groups in mussel matrix (Mytilus edulis). Food Addit. Contam. 25 (8), 1024–1032.

Fux, E., Bire, R., Hess, P., 2009. Comparative accumulation and composition of lipophilic marine biotoxins in passive samplers and in mussels (*M. edulis*) on the West Coast of Ireland. Harmful Algae 8, 523–537.

Hallegraeff, G.M., 1995. Harmful algal blooms: a global overview. In: Hallegraeff, G.M., Anderson, D.M., Cembella, A.D. (Eds.), Manual on Harmful Marine Microalgae. IOC Manuals and Guides, vol. 33. UNESCO, pp. 1–22.

Ito, S., Tsukada, K., 2002. Matrix effect and correction by standard addition in quantitative liquid chromatographic-mass spectrometric analysis of diarrhetic shellfish poisoning toxins. J. Chromatogr. A 943 (1), 39–46.

Lawrence, J.F., Niedzwiadek, B., Menard, C., 2005. Quantitative determination of paralytic shellfish poisoning toxins in shellfish using prechromatographic oxidation and liquid chromatography with fluorescence detection: collaborative study. J. AOAC Int. 88 (6), 1714–1732.

MacKenzie, L., Truman, P., Satake, M., Yasumoto, T., Adamson, J., Mountfort, D., White, D., 1998. Dinoflagellate blooms and associated DSP-toxicity in shellfish in New Zealand. In: Reguera, B., Blanco, J., Fernandez, M.L., Wyatt, T. (Eds.), Harmful Algae Xunta de Galicia and Intergovernmental Oceanographic Commision of UNESCO. pp. 74–77.

MacKenzie, L., Beuzenberg, V., McNabb, P., 2003. Production of gymnodimine by Karenia selliformis (Haywood el al.). In: Steidinger, K.A., Landsberg, J.H., Tomas, C.R., Vargo, G.A. (Eds.), Harmful Algae 2002. Proceedings of the Xth International Conference on Harmful Algae. Florida Fish and Wildlife Conservation Commision and Intergovernmental Oceanographic Commision of UNESCO.

MacKenzie, L., Beuzenberg, V., Holland, P., McNabb, P., Selwood, A., 2004. Solid phase adsorption toxin tracking (SPATT): a new monitoring tool that simulates the biotoxin contamination of filter feeding bivalves. Toxicon 44 (8), 901–918.

MacKenzie, L., 2010. *In situ* passive solid-phase adsorption of micro-algal biotoxins as a monitoring tool. Curr. Opin. Biotechnol. 21, 1–6.

Rundberget, T., Gustad, E., Samdal, I.A., Sandvik, M., Miles, C.O., 2009. A convenient and cost-effctive method for monitoring marine algal toxins with passive samplers. Toxicon 53, 543–550.

Shimizu, Y., 2000. In: Botana, L.M. (Ed.), Seafood and Freshwater Toxins: Pharmacology, Physicology and Detection. Marcel Dekker, New York, pp. 151–162.

Stobo, L.A., Lacaze, J.P., Scott, A.C., Gallacher, S., Smith, E.A., Quilliam, M.A., 2005. Liquid chromatography with mass spectrometry-detection of lipophilic shellfish toxins. J. AOAC Int. 88 (5), 1371–1382.

Turrell, E., Stobo, L., Lacaze, J.P., Bresnan, E., Gowland, D., 2007. Development of an 'early warning system' for harmful algal blooms using solid-phase adsorption toxin tracking (SPATT). In: Oceans 2007 Europe International Conference, Aberdeen Scotland Oceans 2007 – Europe.

Turrell, E., Stobo, L., Lacaze, J.P., 2008. Optimization of hydrophilic interaction liquid chromatography/mass spectrometry and development of solid-phase extraction for the determination of paralytic shellfish poisoning toxins. J. AOAC Int. 91 (6).

Vrana, B., Mills, G.A., Allan, I.J., Dominiak, E., Svensson, K., Knutsson, J., Morrison, G., Greenwood, R., 2005. Passive sampling techniques for monitoring pollutants in water. Trends Anal. Chem. 24 (10).

III.2: Utilización de la adsorción en fase sólida para el seguimiento de la toxina (SPATT) como un sistema para monitorizar la presencia de toxinas lipofílicas en las agua costeras de Europa occidental.

Resumen

La utilización de la fase sólida para el seguimiento de toxinas lipofílicas es una técnica reciente de muestreo pasivo para controlar la presencia de biotoxinas marinas en agua de mar que se ha extendido a varios lugares de todo el mundo. El presente estudio muestra los resultados obtenidos en el trabajo de campo llevado a cabo con bolsas, que contienen resina SP700 distribuidas por varias zonas de producción de moluscos de Europa. Se realizó un seguimiento *in situ* de la dinámica de las toxinas durante los eventos de floraciones algales en un período de tres años en dos áreas de la Costa Oeste de Irlanda y de Escocia y en tres zonas de las Rías Baixas (Galicia, noroeste de España). Se estudió la presencia de OA, DTXs y PTXs en extractos metanólicos de las resinas utilizando como método de análisis el LC-MS/MS. Las tasas de adsorción de las toxinas en las resinas se compararon con la abundancia relativa de las especies productoras de toxinas. Los resultados obtenidos muestran que las toxinas adsorbidas en las bolsas SPATT se correlacionan con la cantidad de células de especies de *Dinophysis* en la columna de agua. El OA se detectó en todos los extractos de resinas y fue la toxina predominante, incluso cuando los dinoflagelados no se detectaron en la columna de agua. Las concentraciones más elevadas de OA se observaron en Cangas y Moaña (Galicia, España) en Agosto y Julio, respectivamente, en el 2007, con niveles que van desde 400 hasta 700 ng de OA/g de resina. Estos resultados, coincidieron con el cierre de polígonos de extracción de moluscos como consecuencia de los resultados positivos en los MBA, realizados en los programas españoles de monitorización de moluscos. Tanto en Clew Bay como Killary Harbour (Irlanda), los mayores niveles de OA fueron obtenidos en Julio del 2009 con una concentración de 300 ng de OA/g de resina y en Loch Ewe (Escocia) en Octubre del 2008 y 2009 con cantidades superiores a 100 ng/g. Otras toxinas lipofílicas como la DTX-1, DTX-2 y PTX-2 también fueron

identificadas en las resinas situadas en todos los puntos de muestreo, a excepción de la DTX-1 que no se detectó en Galicia.

Los resultados de los estudios de campo realizados, demuestran la capacidad de la SPATT como un sistema de muestreo integrado en el tiempo, fiable y sensible para controlar eficazmente la presencia de toxinas DSP y otras toxinas lipofílicas producidas durante los eventos tóxicos de floraciones de algas en las aguas europeas.

Utilisation of solid-phase adsorption toxin tracking (SPATT) as a tool to monitor the presence of lipophilic shellfish toxins in the coastal waters of Western Europe.

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Abstract

Field utilisation of solid-phase adsorption toxin tracking (SPATT) as a recent passive sampling technique to monitor the presence of marine biotoxins in seawater has been carried at several locations across the world. The present study reports on the results of field work involving the use of SPATT bags filled with SP700 resin which monitored *in situ* toxin dynamics during algal bloom events over a three year period at two shellfish production sites off the West Coasts of Ireland and Scotland and at three locations off the Rías Baixas (Galicia, NW Spain). Okadaic acid (OA), dinophysis toxins (DTXs) and pectenotoxins (PTXs) were detected in the SPATT extracts when analysed by liquid chromatography- mass spectrometry. Rates of toxin adsorption onto the resin were compared with the relative abundance of toxin-producing algal species. Results obtained showed that toxins adsorbed onto the SPATT samplers correlated with the amount of *Dinophysis spp.* cells in the water column. Okadaic acid was the predominant toxin detected in all resin extracts, even in the absence of the known causative dinoflagellates. The highest OA concentrations were detected in Cangas and Moaña (Galicia, Spain) in August and July, respectively in 2007 with levels ranging 400-700 ng OA/g of resin. Results from the mouse bioassay used in the Spanish shellfish monitoring programme as the official testing method controlling the presence of diarrhetic shellfish poisoning (DSP) toxins in shellfish led to the closure of these shellfish harvesting areas. Clew Bay as Killary Harbour (Ireland), the highest OA levels were obtained in July 2009 with concentrations around 300 ng OA/g of resin and in Loch Ewe (Scotland) in October 2008 and 2009 with amounts above 100 ng/g. Other

lipophilic toxins such as Dinophysis-1 (DTX1), Dinophysis-2 (DTX2) and pectenotoxin-2 (PTX2) were also identified in the resins deployed in all sampling sites with the exception of DTX1 which was not detected in Galicia.
Results of the field studies reported here demonstrate the ability of SPATT to be a reliable and sensitive time-integrated sampling tool to monitor effectively the presence of DSP toxins and lipophilic shellfish toxins (LST) produced during algal blooms events.

Keywords: Okadaic acid; dinophysis toxins; pectenotoxins; *Dinophysis spp*.; SPATT; SP700.

1. Introduction

The frequency, intensity and geographical distribution of harmful algal bloom (HAB) events have increased over the past decades on a global scale (Hallegraeff 1995). Shellfish aquaculture is particularly affected by HAB events and as a result food safety bodies in shellfish producing countries have implemented phytoplankton and biotoxin monitoring programmes to protect public health and to reduce the cost of limit economic losses for shellfish (Andersen and Throndsen 2003).

Toxins produced by HAB are transferred to food chain and cause numerous human intoxications with different clinical profile. In particular, diarrhetic shellfish poisoning (DSP) caused by Okadaic acid (OA) and Dinophysis toxins (DTXs), is a widely observed syndrome around the world (Tubaro, Sosa et al. 2008). Consumption of shellfish contaminated with high levels of OA-type toxins will result in adverse effects such as gastrointestinal disorder, diarrhea, abdominal cramps, nausea and vomiting (Garcia, Truan et al. 2005). Furthermore, OA and DTX1 have been shown to be tumor promoting substances in animal tests (Manerio, Rodas et al. 2008). Although OA and DTXs have been responsible for most incidents of DSP, pectenotoxins (PTXs) have been also associated, since these toxins usually co-occurs with the OA group and can be produced by the same phytoplankton species (e.g. *Dinophysis spp*.) (Fux, Rode et al. 2008). Human illness has occurred as a result of the ingestion of shellfish contaminated with PTXs (Burgess and Shaw 2001). HABs are largely unpredictable and when they occur in the coastal waters of Western Europe where shellfish aquaculture has developed, this result in severe economic losses for shellfish producers. In the past 25 years ago, shellfish production

areas in Spain, Portugal, France, The Netherlands, Sweden, Norway, Ireland and Italy have been closed for periods sometimes extending from several weeks up to a year due to DSP levels in shellfish above the regulatory threshold (160 µg OA eq./kg) (Van Egmond, Aune et al. 1993; Carmody, James et al. 1996). Many countries now have programs to monitor biotoxin levels in shellfish and control the harvesting of toxic shellfish. There is also a requirement for the monitoring of toxic-producing phytoplankton in the water column (MacKenzie 2010). Although phytoplankton monitoring has proved effective as an early warning method it has some disadvantages. First and foremost, it is difficult to obtain spatially and temporally integrated water samples as they only provide a brief snapshot of the phytoplankton community at one time and place. Furthermore, it is a labour intensive task requiring well trained and dedicated observers (Rundberget, Gustad et al. 2009; MacKenzie 2010) as some phytoplankton species can be difficult to identify. The development of a passive sampling technique called solid phase adsorption toxin tracking (SPATT) for the detection of dissolved lipophilic marine toxins in the water column brings a new environmental tool for scientists who are studying these toxins (MacKenzie, Beuzenberg et al. 2004). The use of passive samplers could be very useful in toxin monitoring programs due to their ability to accumulate toxins onto the receiving phase *in-situ*. They have also the advantage of providing a spatially and temporally integrated response, thereby reducing the number of analyses for long time monitoring (Fux, Marcaillou et al. 2008). Moreover, biotoxin accumulation by the passive sampler resin mimics the action of bivalves filter-feeding on phytoplankton. Finally, SPATT samplers are not affected by heavily polluted waters and will carry on adsorbing toxins while shellfish might have difficulties to survive in harsh environmental conditions. Passive adsorption of dissolved toxins from seawater, coupled to sensitive analytical techniques such as liquid chromatography-mass spectrometry (LC-MS) (Draisci, Palleschi et al. 1999; Goto, Igarashi et al. 2001; Stobo, Lacaze et al. 2005) can provide an easy and rapid testing method for biotoxin monitoring.

An extensive study which span over three years and took place in some coastal waters of Western Europe used the SPATT technique to monitor the presence of DSP and PTX toxins during the spring and summer months when the likelihood of *Dinophysis spp.* blooms was the highest. Results obtained after the

utilisation of SPATT highlights the potential of this technique to provide a useful tool for the screening and early warning of shellfish contamination by lipophilic toxins.

2. Material and methods

2.1. Chemicals

Solvents and chemicals used in this work were of HPLC or analytical grade. Methanol and acetonitrile were purchased from Panreac Quimica S.A. (Barcelona, Spain). Ammonium formate was from Sigma Aldrich (Spain) and formic acid was from Merck (Spain). Ultrapure water was produced from an Arium 611 water purification system (Sartorius, Germany) with a pre-purification filter. Standard solutions of okadaic acid (OA), dinophysis toxin-1 (DTX-1), dinophysis toxin-2 (DTX-2) and pectenotoxin-2 (PTX-2) were provided by Cifga, S.A. (Galicia, Spain).

2.2. Solid phase adsorbent

The resin used in the SPATT samplers, SEPABEADS ® SP700, is an aromatic type adsorbent material based on a cross-linked polystyrene matrix produced by the Mitsubishi Chemical Corporation. SP700 is manufactured in the form of spherical beads (ca. 250µm diameter) with a surface area of 1200 m^2/g and pores volume and radius of 2.2 ml/g and 90 Å respectively (www.diaion.com).

2.3. SPATT monitoring locations

The SPATT bags were deployed at various shellfish harvesting areas in Western Europe. The samplers were made of 100 µm polypropylene mesh sachets (50 mm width) filled with SP700 resin (12 g) and attached held on lines with tie racks at depths of 5 or 7 meters in two sites on the west coast of Ireland (Clew Bay and Killary Harbour), two locations on the west coast of Scotland (Shieldaig and Loch Ewe) and in the Rías Baixas in Galicia (North West of Spain) at three sample points: Cangas, Moaña and Arosa. The study took place during 3 years (2007, 2008 and 2009) at the locations above cited. Deployment and retrieval of the bags was conducted on a weekly basis (Ireland and Scotland) or fortnightly (Galicia). Table 1 summarizes the number of mesh bags

deployed at each location, as well as the frequency and the sampling time during the three years.

2.4. Toxin extraction

Once the SPATT sachets were retrieved from seawater, they were returned to the laboratories and stored at -20 ºC prior to extraction and analysis. The sachets were defrosted at room temperature for 1 hour. The mesh bags were turned inside out and so the SP700 resin could be transferred into 250 mL glass bottles using a funnel. Deionised water was used to rinse the SP700 from the bags into the bottles. A total of 200 mL of water was used to wash and remove the resin for each SPATT bag. The bottles containing the resin were shaken by hand for 1 minute. A resin aliquot (ca. 4.7 g) was poured into empty 25 mL cartridges fitted with 20 µm frits (Ingenieria analitica S.L., Barcelona, Spain). These cartridges are installed on a vacuum manifold Chromabond® from Macherey-Nagel (Düren, Germany). With the resin inside, this was again rinsed with distilled water (100 ± 10 mL) to remove any salts. Interstitial water was gently removed from the resin by increasing the vacuum. Then, the resin was soaked in methanol (10 mL) and mixed for 1 min before it is left for 30 minutes and collected the eluent into a glass bottle. The resin was further eluted with 90 mL methanol and the eluents were pooled in the same bottle. Ten millilitres of the pooled eluent was evaporated to dryness using a centrifugal evaporator RC 10-22 (Jouan, Thermo, electron corporation, Spain) and re-suspended in 500 µL methanol. The sample was filtered through 0.45 µm Ultrafree-MC centrifugal filter devices (Millipore, Spain) and transferred into a vial prior to analysis by LC-MS.

2.5. High performance Liquid Chromatography- Mass Spectrometry (LC-MS) Analysis

HPLC system was comprised of a binary system of LC-10ADvp pumps, an autosampler (SIL-10ADvp) with refrigerated rack, degasser (DGU-14A), column oven (CTO-10ACvp) and a system controller (SCL-10Avp) from Shimadzu (Japan). This system was coupled to a Mass Spectrometer (MS) QTRAP - 2000 equipment from Applied Biosystems (USA), which consists of a hybrid quadrupole-linear ion trap mass spectrometer equipped with atmospheric

pressured ionisation fitted with an electrospray ionisation source (ESI). Nitrogen was supplied by a Nitrocraft $NC_{LC/MS}$ generator (Air Liquide, Spain). Separation of the toxins was achieved using a BDS-Hypersil C8 120 A (50×2 mm; 3µm) column (Phenomenex, USA) attached to a 10×2.1 mm guard cartridge (Thermo, USA). During the analyses, the column oven was set up at 25 ºC and the injection volume was 5 µl. The mobile phases respectively consisted of 100 % water with 2 mM ammonium formate and 50 mM formic acid (Phase A) and acetonitrile:water (95:5) with 2 mM ammonium formate and 50 mM formic acid (Phase B). Analyses were carried out using a linear gradient elution with a constant total flow rate (0.2 mL/min) and a total run time of 14 min. Initial conditions were 30%B, ramping up to 90%B in 8 minutes. These conditions were held for 3 minutes then % B was decreased in 0.5 minutes to 30% and maintained for further 2.5 minutes until the start of the following analysis. Analyst® software was used to control the instrument, process and analyze the data. Sample extracts were analyzed with the mass spectrometer operating in both positive and negative ion modes and the source parameters were as follow: curtain gas, 15; CAD gas, 6; IonSpray voltage, 4000 V; temperature, 450 ºC; gas 1, 50; gas 2, 50. These parameters have been optimised using toxin standards by flow injection analysis. The mass spectrometer was operated in multiple reaction monitoring (MRM), analyzing two product ions per compound: one for quantification (first transition) and the other for confirmation (second transition). In negative ion mode, the transitions selected were: OA and DTX-2: 803.6 > 255.2/113.5; DTX-1: 817.6 > 255.2/113.5. In positive ion mode, the transitions selected for PTX2 were 876.5 > 823.5/213.1.

3. Results

SPATT field trials took place for 3 years in European coastal waters with the aim to assess if this passive sampling technique had the potential to act as an early warning system for marine biotoxins before shellfish contamination. In this study the SPATT bags containing SP700 resin were retrieved on a weekly basis or fortnightly and returned to the laboratories to be extracted and analysed using LC-MS for the detection of DSP and PTXs. In addition, water samples were taken on a weekly basis at the different locations where the SPATT bags were deployed in order to identify the presence of toxic-producing

phytoplankton species (*Dinophysis spp*.). SPATT bags were deployed at Clew Bay, Killary Harbour (West coast of Ireland), Shieldaig and Loch Ewe (West coast of Scotland) and at Cangas, Moaña and Arosa (Rías Baixas, Galicia, Spain). The presence of DSP toxins and PTX-2 was monitored in the SPATT bags during 2007, 2008 and 2009.

3.1. Field trials – Ireland

Clew Bay

During the 2007 sampling period, 14 SPATT bags were weekly deployed from the 13th of August to the 27th of November. *Dinophysis* species were not detected in any of the water samples taken during the monitored period. However detectable amounts of OA, DTX1, DTX2 and PTX2 (figure 1A) were extracted from the resin. During the course of the sampling, OA was the predominant toxin with a median concentration of 34 ng/g, followed by DTX2 and PTX2 which reached a maximum of 36 and 28 ng/g, respectively on the 27th of November. Lower amounts of DTX1 were detected in the resin with a maximum concentration of 8 ng/g on the 17th of September. During the 2008 monitoring period, SPATT bags were deployed for 23 weeks, from the 19th of May to the 11th of November. During this period *D. acuminata* was the only *Dinophysis spp*. detected in the water column. As shown in figure 1B, the dinoflagellate was only detected in July and August at low concentration (40 cells/L). On the 25th of August, the concentration of OA in the resin (90 ng/g) had reached a maximum. Then, the amount of OA declined slowly and it was constant with a mean value of 20 ng/g. Low levels of DTX2 and PTX2 (< LOQ) were also accumulated in the resins. However DTX1 was not detected in the SPATT bags during the 2008 monitored period. The field sampling period in 2009 started on the 25th of May and ended on the 21st of July (figure 1 C). OA, DTX1 and PTX2 levels in the resins were found to remain constant throughout June. During this month, *D. acuminata* was present in the water at 40 cells/L reaching a maximum of 400 cells/L on the 14th of July. One week after (21st of July), an increase of OA and PTX2 concentrations was detected in the resins.

Killary Harbour

During the 2007 monitoring period, the SPATT bags were deployed weekly from the 12th of June to 4th of December (figure 2A). At the beginning of the experiment (June and July), OA and PTX2 were detected in the resins with levels around of 18 and 7 ng/g, respectively. In this period, *D. acuminata* was identified in three water samples with a concentration of 40 cells/L. While DTX1 was not detected in any of the resins, DTX2 it was in the last weeks (from the 23rd October to 4th December). On the 20th of November, the resins accumulated the highest amount of all three toxins in absence of *Dinophysis* species. In 2008, SPATT bags were deployed weekly from the 12th of May to 30th of November. As shown in figure 2B, *D. acuta* was initially detected in May and early June at concentrations of 40 cells/L. The dinoflagellate was not observed until 7th of July when the concentration reached 160 cells/L. Subsequently, there were increases in OA levels in SPATT bags from August to October. During the whole period monitored DTX1 was not detected in the SPATTs however, little amounts of DTX2 and PTX2 (< LOQ) were extracted from the resins. The sampling period in 2009 started on the 1st of June and ended on the 26th of July (figure 2C). Slight increases of OA (from 20 to 60 ng/g) since June to early July were detected in the SPATT bags during the occurrence of *D. acuminata* (80-400 cells/L). In addition, low levels of PTX2 below 10 ng/g were detected in the resins; even traces of DTX1 were identified in the resin on the 28th of June. In the water sample taken on the 5th of July, *D. acuminata* concentration had increased and reached 1960 cells/L while *D. acuta* was also detected in the water (160 cells/L). Subsequently, extraction of the resin contained in the SPATT from the 12th of July showed a large increase in the concentration of OA reaching a maximum of 340 ng/g resin. There was also and increase in PTX2 recovered from the SPATT (23 ng/g) but to a much lesser extent. Levels of *D. acuminata* declined rapidly (120 cells/L) in the subsequent water samples while *D. acuta* concentration remained constant (130 cells/L). Finally, OA concentration in the SPATT collected on the 19th and 26th of July were much lower than in the previous week SPATT (140 and 130 ng/g, respectively) while PTX2 concentrations remained constant.

3.2. Field trials – Scotland

Shieldaig

In 2007, SPATT bags were weekly deployed (29/06 - 10/12). As shown in Figure 3A, *Dinophysis spp.* was detected in July water samples (20-100 cells/L) and a maximum concentration peak was reached on the 16th August (480 cells/L). During August, low cell densities of *Prorocentrum lima* was also detected in two water samples (20-40 cells/L). During the summer months, the levels of OA and PTX2 accumulated in the SPATT oscillated between 20 and 40 ng/g and between 6 and 50 ng/g respectively. Minor quantities of DTX1 and DTX2 were also recovered from the resin with levels below 10 ng/g. From mid-September, the amount of OA and PTX2 recovered weekly from the SPATT decreased with concentrations oscillating between 6 and 20 ng/g while few *Dinophysis spp*. cells (maximum ca. 60 cells/L) were detected in the water samples. It is interesting to note that late in the year, in Mid-November, DTX2 concentration in SPATT reached its maximum value (12 ng/g). During the 2008 monitored period, the SPATT bags were deployed weekly in the water from 8th January to 22nd September (figure 3B). Up to mid-April, OA, PTX2 and low levels of DTX1 and DTX2 (< LOQ) were detected in the SPATTs in the absence of toxin producers. From mid-May, low levels of *Dinophysis spp*. (20 cells/L) were first detected in the water, and then it increased to 100 cells/L on the 4th July returning back to 20-40 until late of sampling. In this period, the toxins concentrations remained constant (< 50 ng/g).

Loch Ewe

During the 2007 monitored period, 51 SPATT bags were deployed weekly from 15th January to 31st December (Figure 4A). Low cell concentrations of *D. acuminata* were detected in the water column during April (20 cells/L). Six weeks later (18/06), the maximum concentration of *D. acuminata* (120 cells/L) was reached in the water. Some morphologically ambiguous *Dinophysis spp.* were also detected in the water (220 cells/L) on the 20th of August. There were no really significant increases of OA and PTX2 concentrations in the SPATT during the summer period. Low levels of DTX1 (< LOQ) were also found in the SPATT in August and September. During this period *D. norvegica* and *P. lima*

were episodically detected in the water column at concentrations of 20-40 cells/L. Extraction of the SPATT retrieved on the 10th of December resulted in higher levels of OA, DTX2 and PTX2 than in the previous SPATTs. Detectable amounts of DTX1 were also accumulated in the same SPATT.

During the first part of the year 2008 (January-July), the SPATT bags accumulated OA, DTX2 and PTX2 (Figure 4B) with concentrations detected no below 25 ng/g (OA). During this period, *D. acuminata*, *P. lima* and some unidentified *Dinophysis spp.* were occasionally detected in low quantities (20 cells/L). From July to late October, these dinoflagellate species were more frequently detected. In addition, *D. norvegica* was also detected (20 cells/L) in a water sample collected on the 18th of August. There was a sudden increase in *Dinophysis spp.* concentration (480 cells/L) in a water sample collected on the 25th of August. During this period, extraction of the resin filled SPATT bags showed higher levels of OA (peak in early October) and PTX2 (peak in August). DTX1 and DTX2 were also detected in the resin extracts at low levels. SPATT bags were also deployed on a weekly basis during 2009 (Figure 4C) and mainly accumulated OA and PTX2 as well as minor amounts of DTX1 and DTX2. There was, on the 20th April a small increase in *Dinophysis spp.* cells detected in the water column but there was no increase in DSP toxins in the SPATT bags subsequently analysed. There were three other episodes in mid-July, end of August and September where the concentration of *Dinophysis spp.* cells increased and reached 220 cells/L, 160 cells/L and 60 cells/L respectively. These phytoplankton episodes were subsequently followed, by increased levels of OA and PTX2 in the resin extracts. From mid-October, the amount of toxins recovered from the SPATT bags reached the low levels found during the winter months. Similarly during the same period, few sporadic *Dinophysis spp.* cells were detected in the water column.

3.3. Field trials – Rías Baixas (Galicia, Spain)

Cangas

Field monitoring sampling was carried out in 2007 from the 24th of July to 26th of November. As shown in Figure 5A, *D. acuminata* was present in the water on the 2nd of July (prior to the bags deployment) at concentrations of 1440 cells/L. *D. rotundata* and an unknown *Dinophysis spp.* were also detected in the water

at this date (40 cells/L). *D. acuminata* reached high concentrations again on the 30th of July (1360 cells/L). Then, the resins indicated high OA levels (775 ng/g) from 24th of July to 6th of August. Concurrently, the shellfish production areas were closed from 23rd of July to 6th of September due to a positive mouse bioassay resulted of shellfish monitoring that was performed during the experiment. From the 10th of September to 3rd of December *D. rotundata*, *D. diegensis*, *D. caudata* and unknown *Dinophysis spp.* were present in the water at concentrations of 40 cells/L. *D. acuta* was also detected but at 120 cells/L while the *D. acuminata* concentrations were more variable. During this period the resins accumulated 192 ng of OA, 176 ng of PTX2 and 41 ng of DTX2 per g of resin while DTX1 was not identified in the monitored period. During the 2008 monitoring field trial**,** the SPATT bags deployment frequency varied from 1 week to 1 month from 3rd of March to 25th of August (figure 5B). At the beginning of the monitored period (March-April), levels of *D. acuminata* oscillated between 40 and 280 cells/L while low levels of toxins were detected in the couple of SPATT deployed during the same period (50 ng/g OA and less than 10 ng/g for DTX2 and PTX2). The first half of May was characterised by a large increase in the number of *D. acuminata* cells present in the water column with a maximum of 1760 cells/L reached on the 19th of May. The SPATT bag deployed during May accumulated much higher levels of OA (204 ng/g). During this period (from 8th of May to 2nd of July), the shellfish production areas were closed as a result of a positive mouse bioassay. From the second half of May, the number of *D. acuminata* cells gradually declined to pre-bloom levels until a smaller bloom started towards the end of July. On the 4th of August, a maximum level of 360 cells/L of *Dinophysis* was reached then quickly receded. Similarly, levels of OA in the SPATT bag deployed over the July period increased (90 ng/g) and it was in the SPATT bag deployed during August that the OA levels plateaued (157 ng/g). DTX2 and PTX2 were also detected in the last SPATT bag but in minor quantities. During this second *D. acuminata* bloom, from 28th of July to late August, the shellfish harvesting areas were closed again as a consequence of a positive mouse bioassay result.

Moaña

During the 2007 monitoring field trial period, four SPATT bags were deployed between the 4th of July and the 28th of November (Figure 6A). *D. rotundata*, *D. acuminata* and a non identified *Dinophysis spp.* were detected in the water column on the 2nd of July at 40, 920 and 120 cells/L respectively. The SPATT bag deployed for almost 3 weeks in July contained high levels of OA (458 ng/g) but DTX1, DTX2 and PTX2 were not detected. During the same month, the shellfish production area was closed as a consequence of a positive mouse bioassay. The cell concentration of *D. acuminata* gradually declined from mid-July and levels during the rest of the year mainly oscillated between 0 and 50 cells/L with the exception of early August and mid-September where spike of *D. acuminata* occurred (200 cells/L and 160 cells/L, respectively). Concentrations of OA in the three SPATT bags deployed during the rest of the year also declined gradually. Traces of PTX2 were detected in the SPATT bag retrieved on the 3rd of September. In the water sample collected on the 17th of September, a non identified *Dinophysis spp.* was detected (320 cells/L) and on the SPATT bag collected on the 28th of November, there was a higher PTX2 concentration (43 ng/g) and also a minor amount of DTX2 (12 ng/g). The field sampling carried out in 2008 started on the 25th of February and ended on the 25th of August (figure 6B). During the first month of sampling, *Dinophysis* species were not detected in the water. However, the resins indicated OA concentrations in the ranging 18 - 43 ng/g and traces of DTX2 and PTX2. On the 12th of May, *D. acuminata* was first detected at 40 cells/L and then it increased until a maximum of 440 cells/L on the 26th of May. This increase was associated with a higher accumulation of OA (200 ng/g) in the resin on the 2nd of June. At the same time, the production area of mussels was closed from 19th of May to 9th of June as consequence of a positive mouse bioassay. Since early of July, *D. acuminata* cells were declined with the OA levels in the resins. Then, in late July and mid-August, *D. acuminata* was detected again in the water at 80 and 40 cells/L, respectively. Subsequently, on the 25th of August, SPATT extract indicated an OA concentration of 117 ng/g and low amounts of DTX2 and PTX2 (< LOQ).

Arosa

In the field sampling period in 2008, SPATT bags were deployed for a 7 week period from 7th of April to 2nd of June and then, the sampling frequency was carried out fortnightly until 19th of December (figure 7A). Low levels of OA (10 ng/g) were detected in April in absence of known causative dinoflagellates. *D. acuminata* was first detected on the 5th and then 26th of May at concentrations of 200 and 360 cells/L, respectively. The appearance and increase in concentration of this organism was followed by higher amounts of OA in the resins immersed in June (65 ng/g) and August (43 ng/g). *D. acuminata* was not detected until September when reached a concentration of 520 cells/L (day 9th). Then it declined quickly one week after and remained at 40 cells/L to late October. In this period (since September to early October) SPATTs accumulated a maximum OA amount of 80 ng/g. In November and December, the OA concentration was around 10 ng/g again. DTX1, DTX2 and PTX2 were not detected through the monitored period.

In the field sampling performed in 2009, the SPATT bags were weekly deployed from the 14th of January to the 18th of June (figure 7B). In the first three months, the resins accumulated trace levels of OA in the absence of toxin producers. On the 31st of March and 7th of April, *D. acuminata* and an unknown *Dinophysis spp.* were present in the water at 40 cells/L. Then, on the 29th of April, *D. acuminata* increased to 240 cells/L and *D. rotundata* was identified in the water at 120 cells/L. The presence of these dinoflagellates species was associated with an OA concentration of 66 ng/g in the resin at the same date. Then, dinoflagellate levels declined until mid-May (40 cells/L) just as the concentration of OA in the SPATTs (20 ng/g). But, on the 19th of May, *D. acuminata* was detected in the water at 120 cells/L and then the resin accumulated an OA amount of 50 ng/g on the 28th of May. *D. acuminata* and OA in the resins declined again to early of June, but on the 9th of this month, the dinoflagellate reached a concentration of 240 cells/L and the SPATT removed from the water on the 18th of June indicated 50 ng/g of OA again. PTX2 was always detected with OA in the resins but in amounts below LOQ while DTX1 and DTX2 were not identified in any of the samplers.

The highest amount of each toxin (OA, DTX1, DTX2 and PTX2) reached during the course of annual monitoring as well as the presence of phytoplankton species during the yearly samplings in Ireland, Scotland and Galicia are summarized in table 2.

The results of the experiments described here for each location demonstrate that the passive solid phase adsorption technique has to provide an early warning of marine biotoxin shellfish contamination. The SPATT bags containing the SP700 resin have shown the ability of responding rapidly to the presence of harmful phytoplankton.

4. Discusion

This paper reports the monitoring of OA, DTXs and PTXs toxins during three years in the West Coast of Ireland, the Northwest highlands of Scotland and in the Rías Gallegas (NW Spain) using SPATT bags. This approach allows correlate phytoplankton appearance with the toxin present in the water and subsequent accumulation in the passive samplers. This is discussed by different locations.

In this study the levels of OA were always allowed for quantification in the SPATT bags even when no *Dinophysis* species were detected in the water.

During the monitoring period in Clew Bay in 2007; OA, DTX1, DTX2 and PTX2 were detected in the resins in the absence of causative dinoflagellates. Similar results were obtained in other field studies in Loch Ewe (Scotland) (Turrell, Stobo et al. 2007), Bantry Bay (Ireland) (Fux, Bire et al. 2009) and also in New Zealand (MacKenzie, Beuzenberg et al. 2004). The detection of toxins without their known producing organism is possibly due to the toxin remaining in the water from a previous toxic event or to the fact that the organism could have been missed during water sampling (Fux, Bire et al. 2009). In 2008 and 2009, this toxin profile was related to the presence of *D. acuminata* in Clew Bay as well as the occurrence of *D. acuta* in Killary Harbour. Similar situation was happened in a field trial in Killary in 2005, an increase of OA and PTX2 concentration in the SPATT samplers was associated to the presence of *D. acuta* (160 cells/L) (Fux, Bire et al. 2009). Indeed in New Zealand, these toxins are always found together in *D. acuta* and *D. acuminata* (MacKenzie, Beuzenberg et al. 2005). Besides, toxin profiles of *D. acuta* have been

described in the coast of Portugal characterized by the presence of DTX2 (Vale and Sampayo 2000) and high levels of OA and DTX2 were found in *D. acuta* from Ireland (Carmody, James et al. 1996; James, Bishop et al. 1997). However DTX2 has not been reported in European *D. acuminata* (MacKenzie, Beuzenberg et al. 2005). Nevertheless, in the study performed here, DTX2 was detected at quantifiable levels in the SPATT bags in Ireland, Scotland and Galicia when *D. acuminata* was present. This could be due to the prior presence of the others *Dinophysis spp*. that could have contaminated to *D. acuminata*.

During the field sampling carried out in Scotland, OA and PTX2 were also the predominant toxins, while DTX1 and DTX2 were detected in minor amounts in the resins. This toxin profile was recurrent in the two locations of sampling (Shieldaig and Loch Ewe). Although a greater proportion of *Dinophysis spp*. *(D. acuminata* and *D. norvegica*) was observed in the phytoplankton population, a low *Prorocentrum lima* cells were detected in the water column. This organism is also a known producer of OA and DTXs (Nascimento, Purdie et al. 2005). Nevertheless, OA and PTX2 have been reported to be generally present in the toxic dinoflagellates *Dinophysis spp*. (Suzuki 2008). So therefore, OA has been identified as the major toxin in *D. norvegica* and *D. acuminata* (Cembella 1989) from the east coast of North America and also high OA concentrations were found in *D. acuminata* from the Bay of Seine (northern France) (Marcaillou, Gentien et al. 2001).

In the monitoring carried out in the Rías Baixas in Galicia (Spain), OA was also the main toxin in the SPATT bags. High *D. acuminata* concentrations (920-1440 cells/L) in the water were followed by an OA concentration increase in July and August 2007. From September to December, *D. acuta*, *D. rotundata, D. caudata, D. diegensis, D. acuminata* and an unknown *Dinophysis spp*. were detected in the water and PTX2 and DTX2 were identified in the resins. PTX2 was found to be the major component of the toxin profile of *D. acuta* and *D. caudata* in the Rías Gallegas in 2001 (Luisa Fernandez, Reguera et al. 2006). Therefore the appearance of PTX2 in the resins could be also associated to the presence of these species. The occurrence of *D. acuminata* in 2008 and 2009 as the predominant *Dinophysis spp.* in the water was related with the OA accumulation in the SPATT bags deployed in the three sampling Galicia

locations: Cangas, Moaña and Arosa. Trace levels of PTX2 and DTX2 were also identified in the resins retrieved from Cangas and Moaña while in Arosa, OA was the only toxin detected. It is possible that DTX2 was associated to the presence of *D. acuta*, althought this organism was just detected at one occasion (28th of April 2008) during sampling in Cangas. Several authors have described wide inter-annual variations in the DTX2 content of *D. acuta* from the Rías Gallegas with OA to DTX2 ratios of 3:1-7:1 (Fernández, Reguera et al. 2001). Previous results on the identification of *Dinophysis* species with the toxin profile here described (OA, DTX2 and PTX2) have been reported in the Rías Gallegas of Vigo and Pontevedra (Luisa Fernandez, Reguera et al. 2006; Villar-Gonzalez, Rodriguez-Velasco et al. 2007). DTX2 and PTX2 outbreaks in Northern Portugal are always related to the occurrence of *D. acuta* and to a minor extent, of *D. fortii* (Vale and Sampayo 2002). In addition, seven species of *Dinophysis* (*D. acuminata*, *D. acuta*, *D. fortii*, *D. rotundata*, *D. mitra*, *D. norvegica* and *D. tripos*) were identified from Japan and Europe to produce either OA or DTX1, or both (Lee, Igarashi et al. 1989). In this study, low levels of DTX1 were detected in Ireland and Scotland; however it was not found evidence of the presence of DTX1 in the Rías Gallegas.

The methanolic extracts obtained from the SPATT bags were checked by LC-MS, it resulted in the definitive identification and accurate quantification of compounds at quantifiable levels. The results shown here have important implications for phycotoxins monitoring programmes. In the Rías Baixas of Galicia, several shellfish harvesting areas affected by the toxic episode were closed for periods due to positive animal bioassay responses. This happened in Cangas and Moaña when OA/DTXs concentrations in mussels exceeded the regulatory limit (160 µg/kg OA equivalents). Similarly in Ireland and Scotland field trials, toxins in the SPATT bags preceded the cumulative concentration of toxins by shellfish. In these cases the shellfish production areas were always opened during the monitoring periods. Even though, the dinoflagellates concentration in the water reached high levels in punctual weeks, these values decreased quickly. It is possible that the mussels did not filter enough toxins to overcome the regulatory limits and lead to closed.

Previously, a synthetic adsorbent resin DIAION HP20, in the form of spherical beads was used for the adsorption of phycotoxins from seawater (MacKenzie,

Beuzenberg et al. 2004; Fux, Marcaillou et al. 2008; Rundberget, Gustad et al. 2009). However, in the present study SP700 was selected because prior experiments demonstrated that this resin appeared to be superior to the HP20 resin for their rapid adsorption of OA from both seawater and deionised water (Turrell, Stobo et al. 2007). The adsorbent resin (SP700) in the bags adsorbs and retains DSP and other lipophilic toxins, and thus provides a time average indicator of toxin concentration in the water. In addition, the SPATT bags have the advantage that directly targets the toxic compounds of interest and is effective where no shellfish exist naturally (i.e. at sentinel sites) because the time and spatially integrated sampling simulates the shellfish uptake (MacKenzie 2010). Another advantage is that SPATT bags are more easily stored and cheaper to transport, and provide matrices relatively clean for the analytical laboratory. The effect of frozen (-20 ºC) storage of the SPATT sachets prior to toxins extraction and analysis was investigated before and freezing did not significantly affect the recovery or determination of toxins from SP700 (Turrell, Stobo et al. 2007). Besides, the materials from which the SPATT bags are constructed are cheap and a significant cost savings within biotoxin monitoring programmes are possible using this method because of the rapid and simple sampling.

In relation with the algal counting as a monitoring tool exists one difficult and is that algal blooms can be short-lived and mobile, and thus occur between algal samplings. Therefore, the passive sampling bags are a valuable tool in that locations, where the algal counts can change quickly from no *Dinophysis*, up to 400 cells/L and back to a few cells/L again during one week. With passive samplers, the water column is continuously being sampled and hence provides an integrated measurement of toxin levels throughout the exposure period. Another problem with algal counting is that can only provide effective monitoring for toxins when the identity of the toxigenic species is known, whereas this information is not necessary when using passive sampling devices (Rundberget, Gustad et al. 2009).

In conclusion, SPATT technique is the very high sensitivity that is achievable which provides the opportunity for lengthy early warning periods. This was apparent during the SPATT field trial when cell numbers were either very low or undetectable. During the SPATT monitoring in the West Coast of Ireland,

Scotland and in the Rías Gallegas, there were good temporal relationships between *D. acuminata* abundance and the concentrations of OA in the SPATT bags. Lower concentrations of PTX2 and also DTX1 and DTX2 could be identified in the SPATTs in presence of other *Dinophysis spp*. In addition, coupling SPATT bags with rapid and field friendly methods for the detection of adsorbed toxins it can provide early warning of impending bloom events.

If the SPATT method is to be used as a regulatory food safety monitoring tool, there is a need for a set of standardized, validated sampling units that will be applicable to all algal-toxin groups. A consensus as to the form and composition of the materials has to be established. However, the present sum of knowledge may already enable SPATT to be used as a supplementary technique to reduce the labour content and costs associated with the traditional shellfish and plankton monitoring methods.

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References

Andersen, P. and Throndsen, J. (2003). Estimating Cell Numbers Manual on Harmful Marine Microalgae G.M. Hallegraeff, D.M. Anderson and A.D. Cembella. Paris, France, Unesco Publishing: 99-130.

Burgess, V. and Shaw, G. (2001). Pectenotoxins-an issue for public health: a review of their comparative toxicology and metabolism. Environ Int 27(4): 275-283.

Carmody, E.P., James, K.J., et al. (1996). Dinophysistoxin-2: the predominant diarrhoetic shellfish toxin in Ireland. Toxicon 34(3): 351-359.

Cembella, A.D. (1989). Ocurrence of okadaic acid a major shellfish toxin in natural populations of *Dinophysis spp*. from the eastern coast of North America. J. Appl. Phycol. 1(307-310).

Draisci, R., Palleschi, L. et al. (1999). New approach to the direct detection of known and new diarrhoeic shellfish toxins in mussels and phytoplankton by liquid chromatography- mass spectrometry. J Chromatogr A 847(1-2): 213-221.

Fernández, M.L., Reguera, B., et al. (2001). Toxin content of *Dinophysis acuminata*, *D. acuta* and *D. caudata* from the Galician Rías Bajas. Harmful Algal Blooms. G.M. Hallegraeff, S.I. Blackburn, C.J. Bolch and R.J. Lewis. Paris, Intergovernmental Oceanographic Commision of UNESCO: 360-363.

Fux, E., Bire, R., et al. (2009). Comparative accumulation and composition of lipophilic marine biotoxins in passive samplers and in mussels (*M. edulis*) on the West Coast of Ireland. Harmful Algae 8: 523-537.

Fux, E., Marcaillou, C., et al. (2008). Field and mesocosm trials on passive sampling for the study of adsorption and desorption behaviour of lipophilic toxins with a focus on OA and DTX1. Harmful Algae 7: 574-583.

Fux, E., Rode, D., et al. (2008). Approaches to the evaluation of matrix effects in the liquid chromatography-mass spectrometry (LC-MS) analysis of three regulated lipophilic toxin groups in mussel matrix (Mytilus edulis). Food Addit. Contam. 25(8): 1024-1032.

Garcia, C., Truan, D., et al. (2005). Metabolic transformation of dinophysistoxin-3 into dinophysistoxin-1 causes human intoxication by consumption of O-acyl-derivatives dinophysistoxins contaminated shellfish. J Toxicol Sci 30(4): 287-296.

Goto, H., Igarashi, T., et al. (2001). Quantitative determination of marine toxins associated with diarrhetic shellfish poisoning by liquid chromatography coupled with mass spectrometry. J Chromatogr A 907(1-2): 181-189.

Hallegraeff, G.M. (1995). Harmful Algal Blooms: A global overview. Manual on Harmful Marine Microalgae. G.M. Hallegraeff, D.M. Anderson and A.D. Cembella, IOC Manuals and Guides No. 33. UNESCO: 1-22.

James, K.J., Bishop, A.G., et al. (1997). Liquid chromatography with fluorometric mass spectrometric and tandem mass spectrometric detection for investigation of the sea-food toxin producing phytoplankton *Dinophysis acuta*. J. Chromatogr. A 777: 213-221.

Lee, J.S., Igarashi, T.T., et al. (1989). Determination of diarrhetic shellfish toxins in various dinoflagellate species. J. Appl. Phycol. 1: 147-152.

Luisa Fernandez, M., Reguera, B., et al. (2006). Pectenotoxin-2 in single-cell isolates of *Dinophysis caudata* and *Dinophysis acuta* from the Galician Rias (NW Spain). Toxicon 48(5): 477-490.

MacKenzie, L., Beuzenberg, V., et al. (2004). Solid phase adsorption toxin tracking (SPATT): a new monitoring tool that simulates the biotoxin contamination of filter feeding bivalves. Toxicon 44(8): 901-918.

MacKenzie, L., Beuzenberg, V., et al. (2005). Pectenotoxin and okadaic acid-based toxin profiles in *Dinophysis acuta* and *Dinophysis acuminata* from New Zealand. Harmful Algae 4: 75-85.

MacKenzie, L.A. (2010). *In situ* passive solid-phase adsorption of micro-algal biotoxins as a monitoring tool. Current Opinion in Biotechnology 21: 1-6.

Manerio, E., Rodas, V.L., et al. (2008). Shellfish consumption: a major risk factor for colorectal cancer. Med Hypotheses 70(2): 409-412.

Marcaillou, C., Gentien, P., et al. (2001). *Dinophysis acuminata* distribution and specific toxin content in relation to mussel contamination. Harmful Algal Blooms 2000. G.M. Hallegraeff, S.I. Blackburn, C.J. Bolch and R.J. Lewis. Oeanographic Commission of UNESCO: 356-359.

Nascimento, S.M., Purdie, D.A., et al. (2005). Morphology, toxin composition and pigment content of *Prorocentrum lima* strains isolated from a coastal lagoon in southern UK Toxicon 45: 633-649.

Rundberget, T., Gustad, E., et al. (2009). A convenient and cost-effctive method for monitoring marine algal toxins with passive samplers. Toxicon 53: 543-550.

Stobo, L.A., Lacaze, J.P., et al. (2005). Liquid chromatography with mass spectrometry-detection of lipophilic shellfish toxins. J AOAC Int 88(5): 1371-1382.

Suzuki, T. (2008). Chemistry, Metabolism, and Chemical Detection Methods of Pectenotoxins. Seafood and Freshwater Toxins: Pharmacology, Physiology, and Detection. L.M. Botana. Boca Raton, FL, CRC Press. Taylor & Francis Group: 343-380.

Tubaro, A., Sosa, S., et al. (2008). Pharmacology and Toxicology of Diarrheic Shellfish Toxins. Seafood and Freshwater Toxins: *Pharmacology, Physiology, and Detection*, 2nd edition. L.M. Botana. Boca Raton, FL, Taylor and Francis Group: 229-253.

Turrell, E., Stobo, L., et al. (2007). Development of an 'early warning system' for harmful algal blooms using solid-phase adsorption toxin tracking (SPATT). Oceans 2007 Europe International Conference, Aberdeen SCOTLAND OCEANS 2007 - EUROPE.

Vale, P. and Sampayo, M.A.M. (2000). Dinophysistoxin-2: a rare diarrhoeic toxin associated with *Dinophysis acuta*. Toxicon 38: 1599-1606.

Vale, P. and Sampayo, M.A.M. (2002). Pectenotoxin-2 seco acid, 7-epi-pectenotoxin-2 seco acid and pectenotoxin-2 in shellfish and plankton from Portugal. toxicon 40: 979-987.

Van Egmond, H.P., Aune, T., et al. (1993). Paralytic and diarrhoeic shellfish poisons: occurrence in Europe, toxicity, analysis and regulation. J. Nat. Toxins 2: 41-83.

Villar-Gonzalez, A., Rodriguez-Velasco, M.L., et al. (2007). Lipophilic toxin profile in Galicia (Spain): 2005 toxic episode. Toxicon 49(8): 1129-1134.

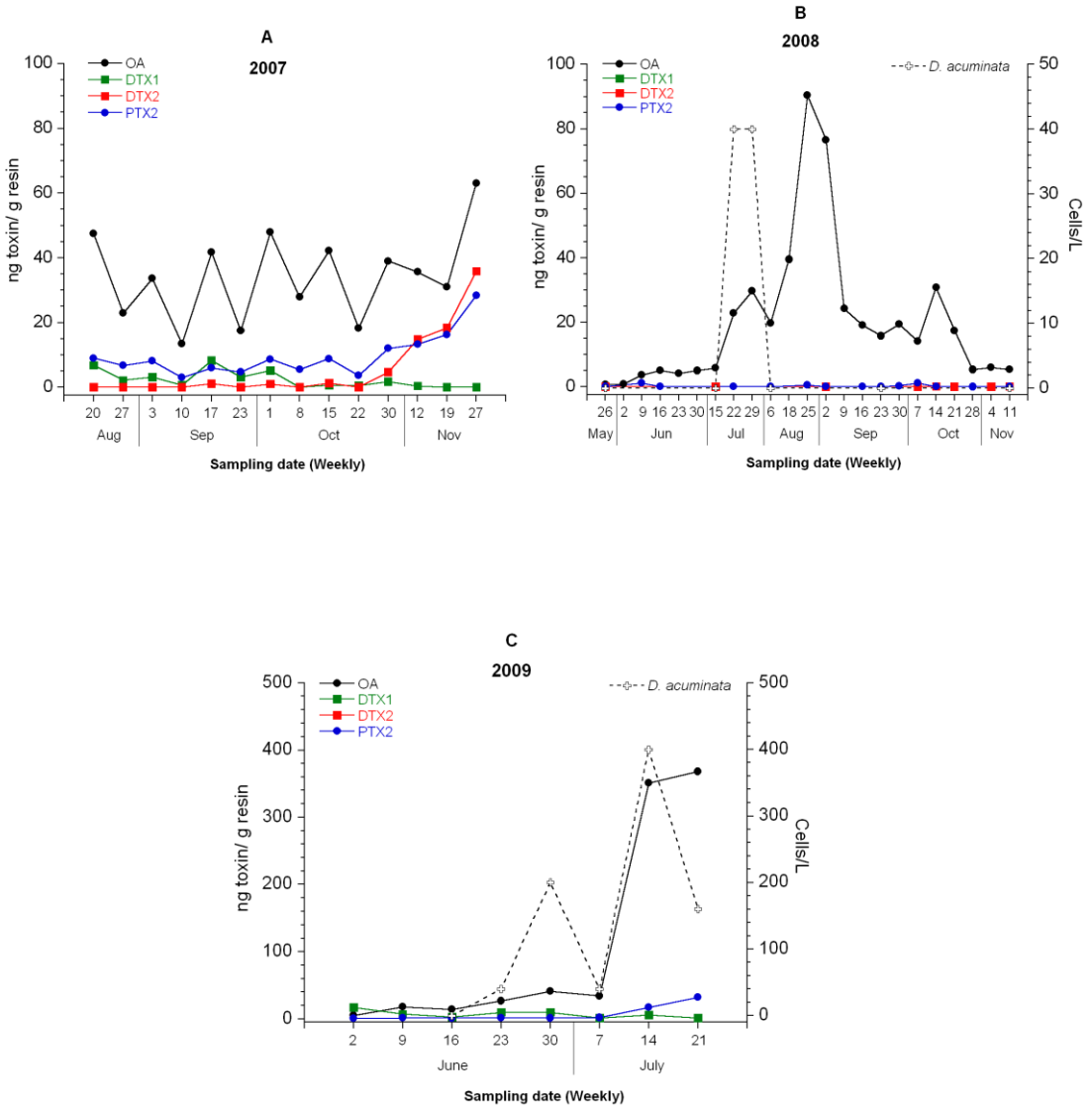

Figure 1: Toxins and phytoplankton results in Clew Bay (West Coast of Ireland). (A): concentrations of OA, DTX1, DTX2 and PTX2 in the SPATT bags (from 13th of August to 27th of November 2007). (B): concentrations of OA, DTX2 and PTX2 in the SPATT bags (from 19th of May to 11th of November 2008) and concentrations of *D. acuminata* in the water column. (C): concentrations of OA, DTX1 and PTX2 in the SPATT bags (from 25th of May to 21st of July 2009) and concentrations of *D. acuminata* in the water column.

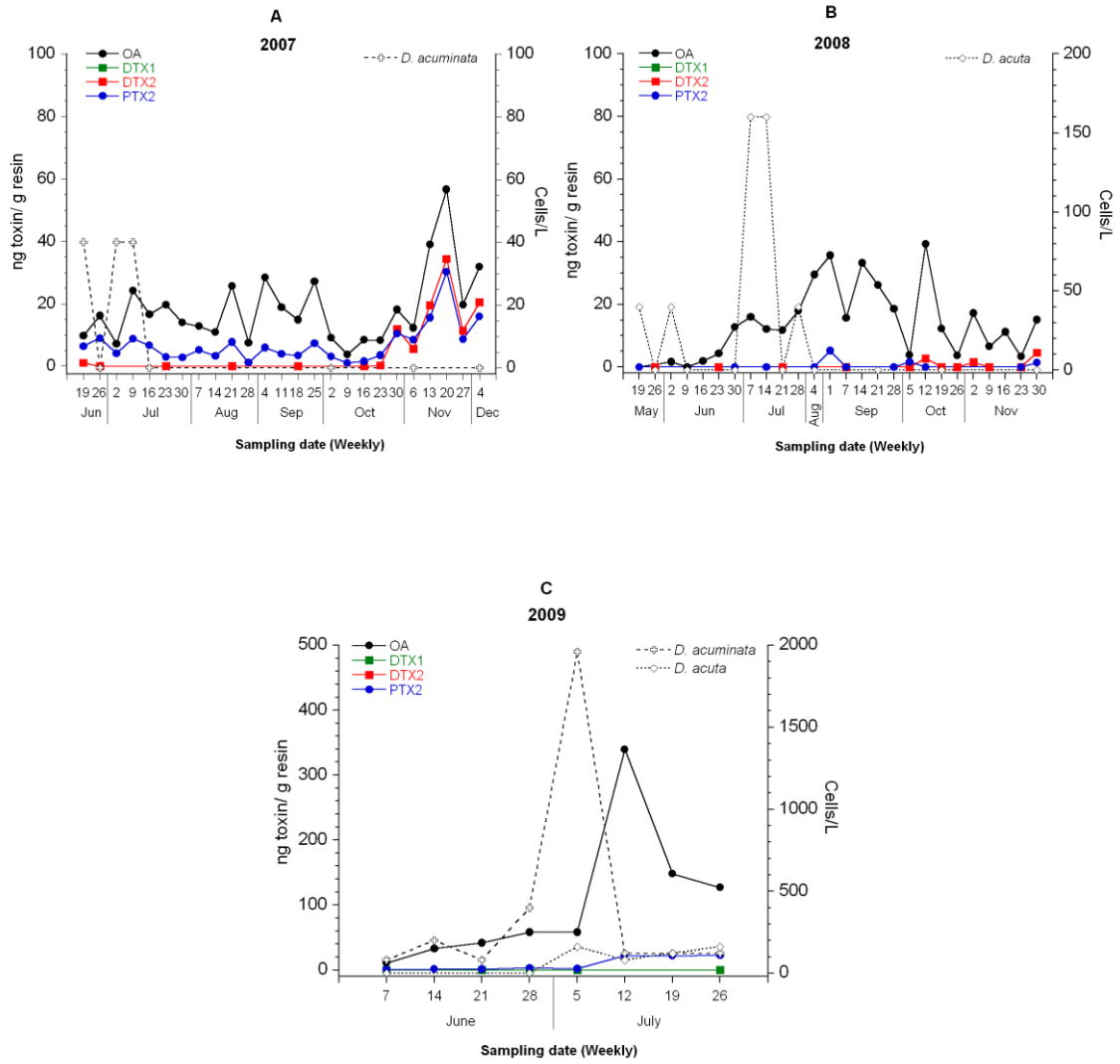

Figure 2: Toxins and phytoplankton results in Killary Harbour (West Coast of Ireland). (A): concentrations of OA, DTX2 and PTX2 in the SPATT bags (from 12th of June to 4th of December 2007) and concentrations of *D. acuminata* in the water column. (B): concentrations of OA, DTX2 and PTX2 in the SPATT bags (from 12th of May to 30th of November 2008) and concentrations of *D. acuta* in the water column. (C): concentrations of OA, DTX1 and PTX2 in the SPATT bags (from 1st of June to 26th of July 2009) and concentrations of *D. acuminata* and *D. acuta* in the water column.

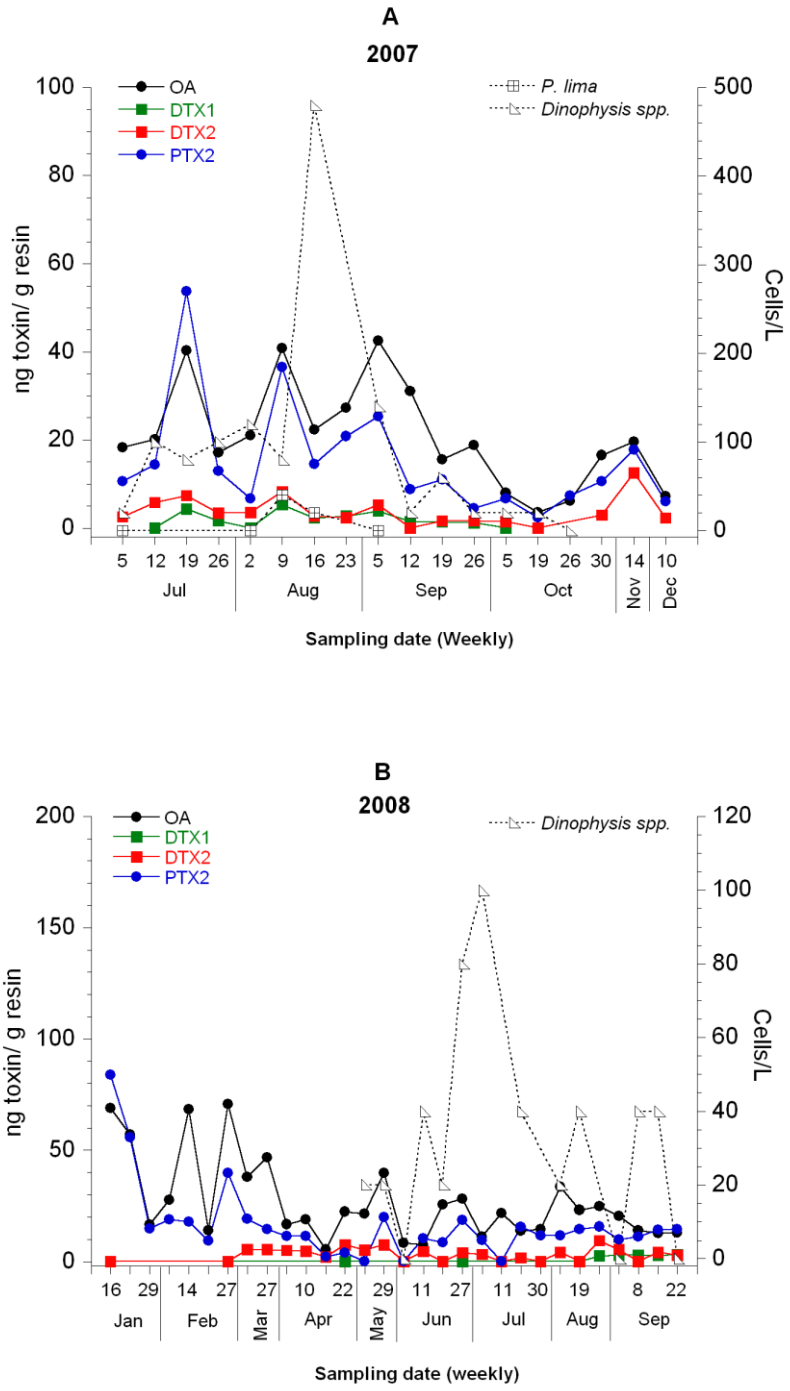

Figure 3: Toxins and phytoplankton results in Shieldaig (West Coast of Scotland). (A): concentrations of OA, DTX1, DTX2 and PTX2 in the SPATT bags and concentrations of *Dinophysis spp*. and *P. lima* in the water column from 29th of June to 10th of December 2007 and (B): from 8th of January 2008 to 22nd of September 2009.

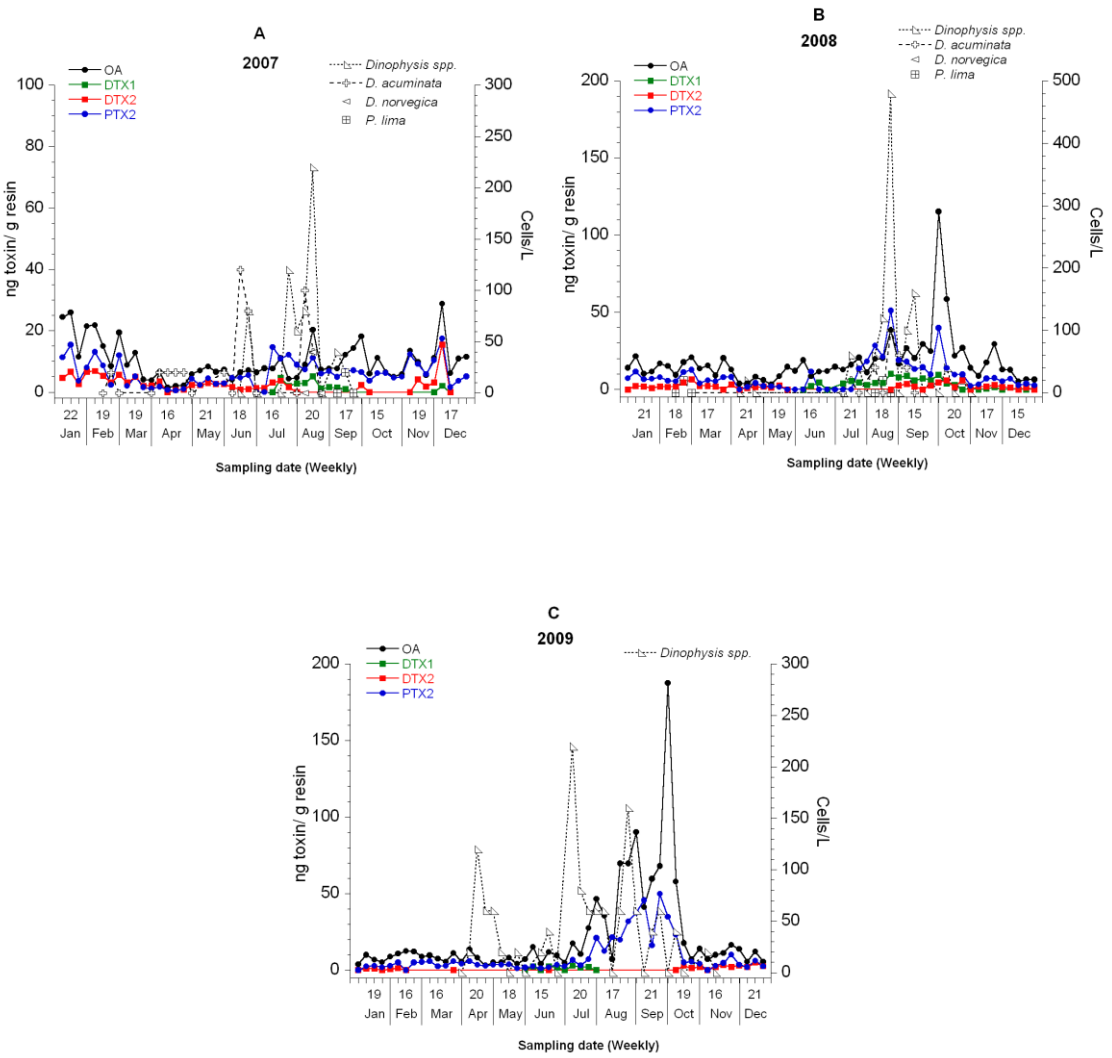

Figure 4: Toxins and phytoplankton results in Loch Ewe (West Coast of Scotland). (A): concentrations of OA, DTX1, DTX2 and PTX2 in the SPATT bags and concentrations of *Dinophysis* species and *P. lima* in the water column from 15th of January to 31st of December 2007; (B): from 7th of January to 29th of December 2008 and (C): from 5th of January 2008 to 28th of December 2009.

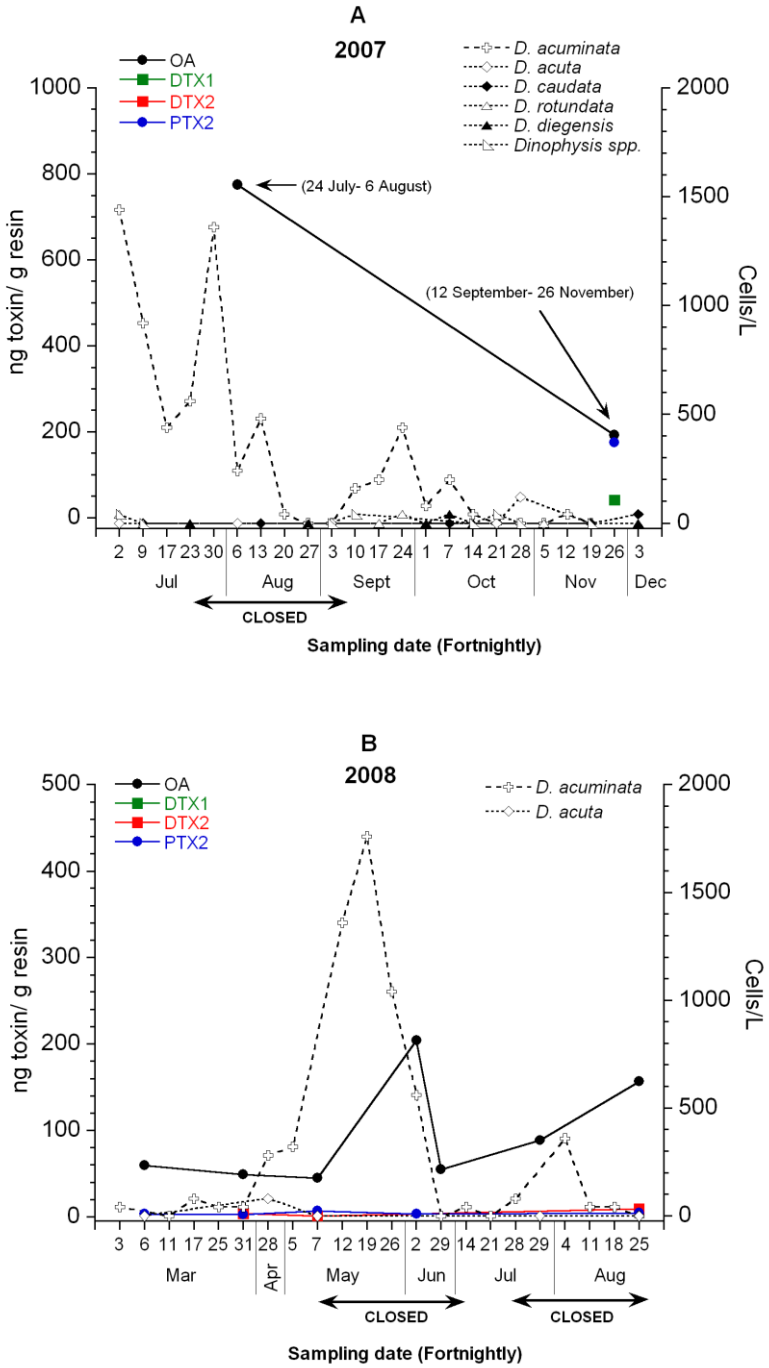

Figure 5: Toxins and phytoplankton results in Cangas (Rías Baixas, Galicia, Spain). (A): concentrations of OA, DTX2 and PTX2 in the two SPATT bags deployed from 24th of July to 6th of August and from 12th of September to 26th of November 2007 and concentrations of *Dinophysis* species in the water column (from 2nd of July to 3rd of December). (B): concentrations of OA, DTX2 and PTX2 in the SPATT bags and concentrations of *D. acuminata* and *D. acuta* in the water column (from 3rd of March to 25th of August 2008).

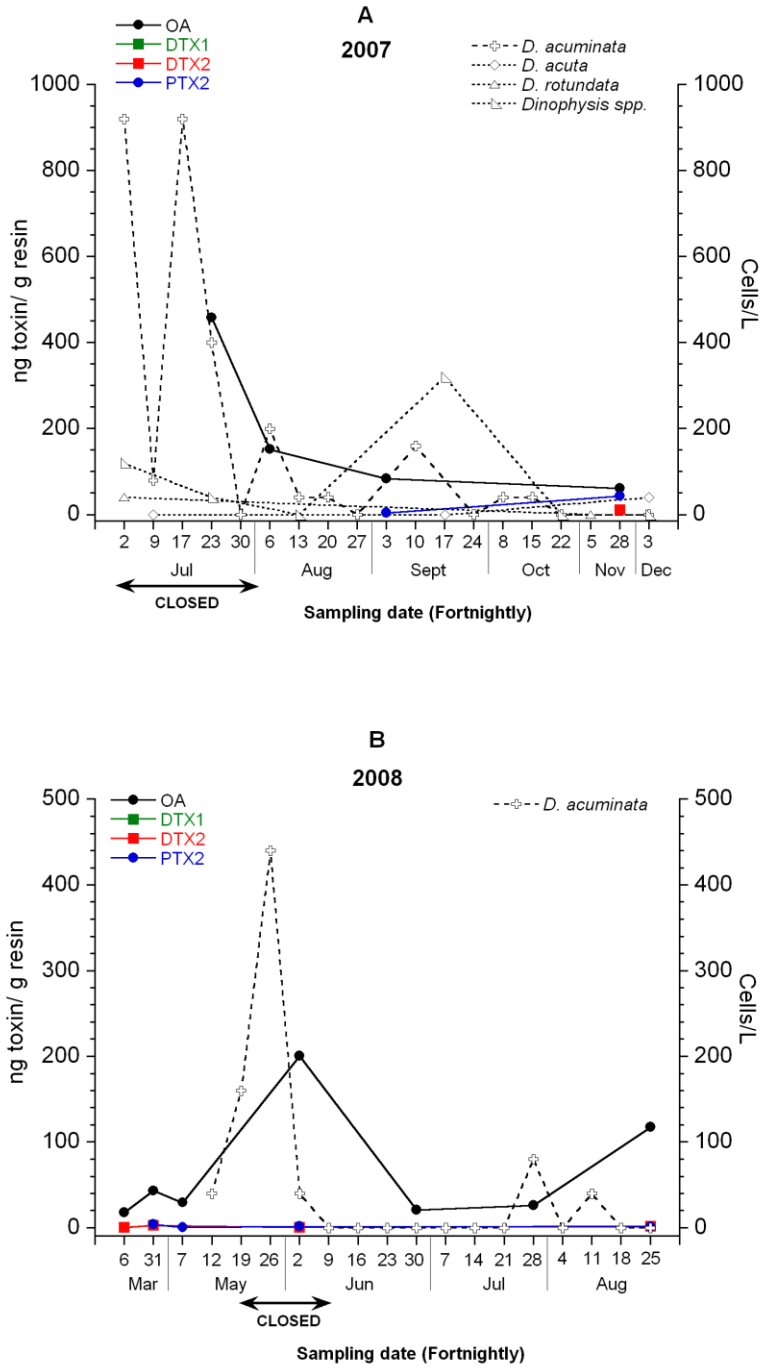

Figure 6: Toxins and phytoplankton results in Moaña (Rías Baixas, Galicia, Spain). (A): concentrations of OA, DTX2 and PTX2 in the SPATT bags deployed from 4th of July to 28th of November 2007 and concentrations of *Dinophysis* species in the water column (from 2nd of July to 3rd of December 2007). (B): concentrations of OA, DTX2 and PTX2 in the SPATT bags and concentrations of *D. acuminata* in the water column (from 25th of February to 25th of August 2008).

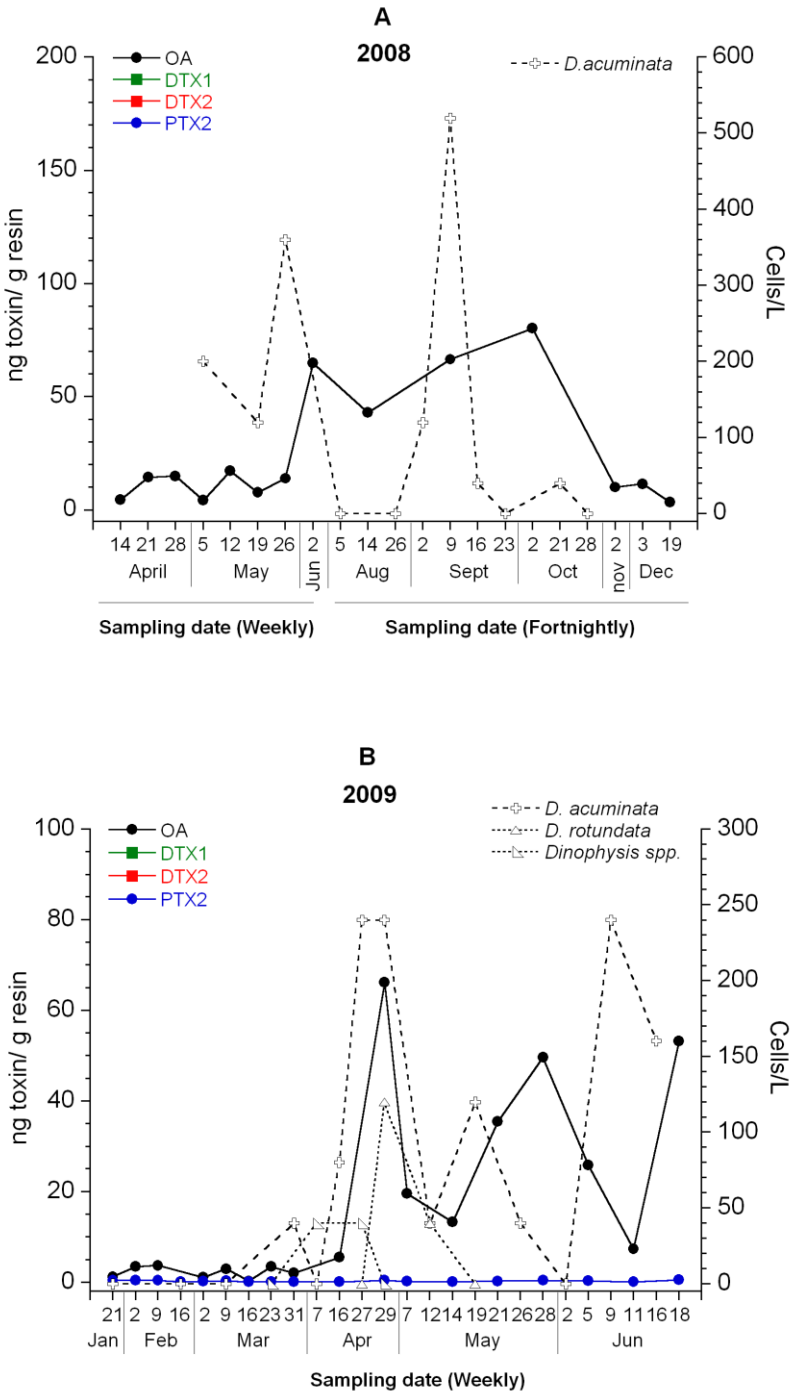

Figure 7: Toxins and phytoplankton results in Arosa (Rías Baixas, Galicia, Spain). (A): concentrations of OA in the SPATT bags and concentrations of *D. acuminata* in the water column (from 7th of April to 19th of December 2008). (B): concentrations of OA and PTX2 in the SPATT bags and concentrations of *Dinophysis spp.* in the water column (from 14th of January to 18th of June 2009).

Table 1: Number of mesh bags deployed in each shellfish harvesting area of Ireland, Scotland and Galicia Coasts and the frequency and sampling time during 2007, 2008 and 2009. (* Frequency of deployment and retrieval of mesh bags; — No deployment).

		Ireland		**Scotland**		**Galicia**		
		Clew Bay	Killary Harbour	Shieldaig	Loch Ewe	Cangas	Moaña	Arosa
2007	Time-scale	13 Aug-27 Nov	12 Jun-4 Dec	29 Jun-10 Dec	15 Jan-31 Dec	24 Jul-26 Nov	4 Jul-28 Nov	—
	Frequency*	weekly	weekly	weekly	weekly	fortnightly	fortnightly	—
	Nº mesh bags	14	25	18	51	2	4	—
2008	Time-scale	19 May-11 Nov	12 May-30 Nov	8 Jan-22 Sep	7 Jan-29 Dec	3 Mar-25 Aug	25 Feb-25 Aug	7 April-19 Dec
	Frequency*	weekly	weekly	weekly	weekly	fortnightly	fortnightly	fortnightly
	Nº mesh bags	23	26	30	52	7	7	14
2009	Time-scale	25 May-21 Jul	1 Jun-26 Jul	—	5 Jan-28 Dec	—	—	14 Jan-18 Jun
	Frequency*	weekly	weekly	—	weekly	—	—	fortnightly
	Nº mesh bags	8	8	—	52	—	—	17

Table 2: Ireland, Scotland and Galicia results – Highest amount detected of each toxin during the annual monitoring and presence of phytoplankton species in each year. (* means: Limit of quantification for each toxin in ng/g; ** means: Morphologically ambiguous *Dinophysis spp*).

		IRELAND								SCOTLAND						GALICIA, SPAIN							
		Clew Bay			Killary Harbour			Shieldaig			Loch Ewe			Cangas			Moaña			Arosa		
		2007	2008	2009	2007	2008	2009	2007	2008	2007	2008	2009	2007	2008	2007	2008	2007	2008	2007	2008	2008	2009
		Peak (month)	Peak (month)	Peak (month)	Peak (month)	Peak (month)	Peak (month)	Peak (month)	Peak (month)	Peak (month)	Peak (month)	Peak (month)	Peak (month)	Peak (month)	Peak (month)	Peak (month)	Peak (month)	Peak (month)	Peak (month)	Peak (month)	Peak (month)	Peak (month)	Peak (month)
Toxins (ng/g resin)	OA (6.5)*	63.0 27-Nov	90.4 25-Aug	368.1 21-Jul	56.7 20-Nov	39.3 12-Oct	339.4 12-Jul	42.5 9-Aug	70.8 27-Feb	28.8 10-Dec	115.4 6-Oct	187.5 5-Oct	775.0 6-Aug	204.3 2-Jun	458.3 23-Jul	200.5 2-Jun	80.3 2-Oct	66.1 29-Abr				
	DTX1 (7.3)*	8.2 17-Sep	—	17.3 2-Jun	—	—	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	—	—	—	—	—	—				
	DTX2 (9.8)*	35.8 27-Nov	< LOQ	—	34.4 20-Nov	< LOQ	—	12.5 14-Nov	< LOQ	15.5 10 dec	< LOQ	< LOQ	41.42 26-Nov	10.0 25-Aug	12.0 28-Nov	< LOQ	—	—				
	PTX2 (5.3)*	28.3 27-Nov	< LOQ	32.2 21-Jul	30.3 20-Nov	< LOQ	22.8 12-Jul	53.7 19-Jul	83.8 8-Jan	17.5 10-Dec	51.1 25-Aug	49.8 28-Sep	175.8 26-Nov	7.0 7-May	42.92 28-Nov	< LOQ	—	< LOQ				
Dinoflagellates species	*D. acuminata*		X	X	X		X			X	X		X	X	X	X	X	X				
	D. acuta					X	X						X	X	X							
	D. norvegica									X	X											
	D. caudata												X									
	D. rotundata												X		X							
	D. diegensis												X									
	D. spp. **							X	X	X	X	X	X		X							
	P. lima							X		X	X											

4. Discusión

Se acepta generalmente que las HABs están aumentando en frecuencia, intensidad y duración en todos los ambientes acuáticos en una escala global. Estos episodios son responsables de importantes pérdidas en el sector acuícola, pesquero y marino mundial [390, 391]. La causa de esta expansión podría ser el calentamiento global, sin embargo hay relativamente pocos trabajos que expliquen esta conexión [392-394]. El principal problema es la dificultad de separar la influencia del cambio climático de otros factores antropogénicos, como la eutrofización, la introducción de aguas de lastre de los barcos o el incremento del tráfico marítimo, que se sabe que contribuyen a la aparición de algunas floraciones de algas tóxicas. Teniendo en cuenta que el crecimiento del fitoplancton está fuertemente determinado por la temperatura, la luz y la disponibilidad de nutrientes, no es de extrañar que las interacciones clima – océano den como resultado cambios en el fitoplancton que pueden influir en el desarrollo de las HABs [395, 396]. En cualquier caso, la consecuencia final es que especies de microalgas tóxicas típicas de un área geográfica, aparecen con más frecuencia o bien se extienden a otras regiones donde antes no se detectaban. Por ejemplo, los dinoflagelados del género *Gambierdiscus*, endémicos de las regiones tropicales y subtropicales con arrecifes coralinos, pueden aparecer en otras latitudes por un aumento en la temperatura del agua [397]. El resultado de esta aparición da lugar a CFP en zonas geográficas distintas [398]. Asimismo los dinoflagelados del género *Alexandrium*, productor de toxinas PSP, tienen hoy en día una distribución global al igual que las toxinas DSP, las YTXs, PTXs y los AZAs. Las toxinas NSP están principalmente limitadas a Estados Unidos y Nueva Zelanda, mientras que las toxinas lipofílicas ocurren más frecuentemente en Europa [8].

Por otro lado, la apertura del canal de Suez en 1869 ha favorecido la invasión de muchas especies marinas procedentes del océano Índico, a través del mar Rojo que se han establecido recientemente en el Mediterráneo [399]. La migración de estas especies, peces u otros animales marinos, está dando lugar a intoxicaciones que anteriormente se asociaban a regiones tropicales. Como es el caso de las intoxicaciones producidas por las TTXs. Estas intoxicaciones ocurren frecuentemente en países asiáticos debido al consumo de peces globo o pequeños gasterópodos contaminados con estas toxinas [322, 342]. Sin

embargo, en la presente tesis doctoral se describe el primer caso de intoxicación por TTX en las costas europeas. La TTX y el análogo 5,6,11-trideoxyTTX se detectaron en la glándula digestiva de una caracola de la especie *Charonia lampas* además de en los fluidos de un paciente intoxicado por el consumo de esta caracola. Asimismo otras intoxicaciones han ocurrido recientemente en el mar Mediterráneo debido al consumo de peces globo tóxicos. Estos peces posiblemente se asentaron en el Mediterráneo como consecuencia de la migración lessepsiana [332]. Estos hallazgos alertan sobre la posibilidad de que los organismos (microalgas, bacterias…) implicados en la producción de las TTXs se están extendiendo por zonas menos cálidas y sus toxinas son transferidas a través de la cadena alimentaria marina.

La captura de peces o gasterópodos, contaminados con toxinas de aguas tropicales y subtropicales en el océano Atlántico o en el mar Mediterráneo tienen gran importancia epidemiológica. Este fenómeno podría suponer un indicio de las posibles consecuencias del calentamiento global sobre la seguridad alimentaria. Las intoxicaciones, que antes se diferenciaban por el área geográfica en la que sucedían son actualmente más difíciles de distinguir, ya que aparecen nuevas toxinas, aumenta el número de análogos de las ya conocidas y además hay diferentes toxinas que producen los mismos efectos tóxicos, como por ejemplo, STXs y TTXs. Estas toxinas tienen una estructura similar [277, 400], un mecanismo de acción común [356, 357] y causan síntomas similares en una intoxicación. De hecho, han sido identificadas juntas en una variedad de organismos [209, 335, 401, 402]. Y por ello, una intoxicación debida a TTXs puede ser erróneamente atribuida a toxinas PSP. En este sentido, el diagnóstico clínico de la intoxicación por *Charonia lampas* ocurrida en Málaga, que se describe en el presente trabajo se debió inicialmente a la presencia de toxinas PSP en la caracola, ya que estas toxinas aparecen frecuentemente en aguas más frías [403]. Además, el MBA dio positivo para estas toxinas. Sin embargo los análisis realizados por HPLC-FLD mostraron que la STX no era la toxina implicada en la intoxicación. Finalmente los análisis por LC-MS/MS confirmaron la presencia de TTXs en las muestras. Se han descrito resultados similares utilizando estas tecnologías para informar sobre otros casos de intoxicación por TTX después del consumo de pescado [371, 404]. Así pues, la investigación se centra cada vez más en el uso de

métodos analíticos adecuados que permitan aislar e identificar las toxinas y así poder clasificarlas dentro de un mismo grupo [209, 369, 370, 405].

Las distintas condiciones ecológicas y climáticas determinan perfiles de toxinas distintos. Esto es un problema a la hora de determinar la toxicidad de una muestra debido a que hay toxinas que son más toxicas que otras. Por ello, es necesario complementar los métodos analíticos (HPLC, LC-MS) con los métodos funcionales (MBA, ELISA, entre otros). Los primeros permiten conocen el perfil de toxinas real de una muestra mientras que los segundos nos informan de la toxicidad total de la misma. En este sentido, la combinación de estudios instrumentales, toxicológicos y cinéticos se utilizó en la investigación realizada con las muestras de *Charonia lampas*. Y así se observó que aunque el análogo 5,6,11-trideoxyTTX se detectó en una cantidad 3 veces superior a la TTX en todas las muestras, su toxicidad fue mucho menor. Lo que ya se había apuntado anteriormente [406].

El hecho de que la TTX aparezca con más frecuencia en Europa, y que cada vez se conozcan más análogos de esta toxina, hace que el desarrollo de métodos analíticos que permitan la identificación de todos los análogos presentes en una muestra cobre mucha importancia. Recientemente estas toxinas han sido detectadas en los tejidos de peces globo de la especie *Lagocephalus sceleratus* capturados en el mar Egeo, Grecia [328]. Por eso, se desarrolló un método de LC-MS/MS con el que se consigue identificar y cuantificar los análogos de TTX presentes en las muestras mediante la monitorización de reacciones múltiples (MRM). La identificación de los análogos se basa en los espectros iónicos obtenidos a partir de la fragmentación paterna de cada molécula y la comparación de estos con los descritos por otros autores [367]. Esta técnica ofrece una herramienta sensible que permite detectar varias toxinas en un solo análisis y en cantidades en el rango nanomolar [209, 369, 370, 405].

No todas las toxinas PSP muestran la misma toxicidad y los perfiles de estas toxinas son diferentes en muestras de dinoflagelados y en muestras de moluscos debido a las conversiones que se producen durante su metabolización. Actualmente en la UE, los métodos oficiales para la detección de estas toxinas son el MBA [286] y el método de HPLC-FLD, con oxidación pre-columna de Lawrence [288]. El trabajo llevado a cabo en la presente tesis

doctoral se centró en la detección de las toxinas PSP en diferentes muestras de moluscos y dinoflagelados utilizando el método de Lawrence. Debido a los inconvenientes encontrados en este método [317] las muestras también se analizaron por el método de HPLC-FLD con oxidación post-columna basado en el método descrito por Oshima [310]. Este método ha sido utilizado durante años por muchos laboratorios para la detección de toxinas PSP en sus controles rutinarios. Hoy en día el método de Oshima está sometido a continuas variaciones y se sigue utilizando como una alternativa al MBA aunque no oficialmente [239, 311, 314, 320, 407]. El objetivo de utilizar los métodos de Lawrence y Oshima en la detección de toxinas PSP fue evaluar las ventajas e inconvenientes de ambos métodos cuando se analizan muestras con diferentes perfiles de toxinas.

Además de la detección de las toxinas, es importante distinguir entre microalgas tóxicas y no tóxicas dentro de una misma especie y el hecho de que especies tóxicas lo sean en una región y no en otra cercana. Como ocurre con los dinoflagelados del género *Alexandrium* en las costas de Irlanda. [241, 378]. Ya que la monitorización del fitoplancton es una herramienta muy utilizada en el control oficial. En este sentido, parte de la investigación realizada en el presente trabajo se centró en el estudio de las distribuciones de *A. minutum* y *A. tamarense* en las costas de Irlanda. Se estudiaron muestras recogidas durante una floración de especies de *Alexandrium* en el verano del 2006 en Cork Harbour. Estas muestras se analizaron por el método de HPLC-FLD de Oshima con el objetivo de obtener un perfil tóxico detallado, ya que este método es capaz de separar todas las toxinas PSP. De esta forma se identificó la GTX3 como la toxina dominante en *A. minutum* además de la detección de las Cs que no se habían observado con anterioridad en Cork. Sugiriendo que estas toxinas pueden ser sintetizadas bajo ciertas condiciones medioambientales o bien podría tratarse de una subpoblación de *A. minutum* que produce las Cs además de GTX2,3.

Un sistema de alerta temprana de la aparición de las HABs y la contaminación con biotoxinas es importante para proteger a los consumidores de productos marinos. Muchos países tienen hoy en día programas de monitorización del fitoplancton así como controles de las toxinas marinas en los moluscos. Sin embargo, a veces resulta difícil seguir la dinámica de una floración (formación,

duración y dispersión), debido a las variaciones de toxicidad de las microalgas y la convivencia de especies de algas tóxicas con no tóxicas. Como consecuencia se pueden producir alertas innecesarias. Por lo tanto, la investigación se centra cada vez más en el desarrollo de métodos que permitan el seguimiento de la toxina *in situ* en la columna de agua mediante la utilización de muestreadotes pasivos.

Desde que se desarrolló el sistema SPATT [375], se ha probado la técnica en distintos estudios y además se han experimentado diversos sustratos de adsorción. Sin embargo la mayoría se centraron en la detección de toxinas del grupo del OA, PTXs, YTXs y AZAs [408-412]. En vista de esto, parte de los resultados mostrados en esta tesis, se basa en el estudio de la adsorción pasiva en fase sólida para las toxinas PSP y también para toxinas lipofílicas disueltas en agua de mar. Este trabajo se realizó en el contexto del proyecto europeo SPIES-DETOX [389]. Se evaluó la adsorción y desorción de estas toxinas sobre los poros de la resina sintética SP700 y el polímero CDP. Ambos sustratos fueron examinados anteriormente en otros trabajos en donde mostraron su afinidad por estas toxinas [413, 414]. Los resultados de los experimentos demostraron que tanto el polímero CDP como la resina SP700 adsorben un amplio rango de toxinas PSP además de OA y DTXs, mostrando el polímero CDP más afinidad por las toxinas PSP. Este sistema de adsorción pasiva, utilizando CDP y/o SP700 podría proporcionar una información detallada mediada en el tiempo sobre el perfil de toxinas hidrofílicas presentes en la columna de agua.

Conjuntamente con los experimentos anteriores, se realizaron estudios de campo, utilizando la resina SP700 como un muestreador pasivo para monitorizar la presencia de toxinas lipofilicas en aguas de las costas europeas. Este trabajo, basado en la técnica SPATT se llevo a cabo durante tres años en diferentes localizaciones de Irlanda, Escocia y España. El sistema de bolsas SPATT permitió establecer una buena correlación entre la cantidad de células de los dinoflagelados productores de las toxinas y la cantidad de estas toxinas en las resinas, incluso aún cuando estas estaban disueltas en pequeñas cantidades en la columna de agua. La técnica SPATT, acoplada con métodos de detección rápidos y sensibles que permitan determinar la concentración de las toxinas, podría ser empleada por la industria de la acuicultura y por las

autoridades reguladoras como una tecnología de alerta temprana para predecir los cierres de las zonas de recolección de moluscos como consecuencia del desarrollo de las HABs.

Como conclusión y debido al continuo incremento de las HABs es necesario aumentar los controles de vigilancia, tanto de las microalgas tóxicas como de las toxinas marinas. Así como desarrollar métodos de detección que permitan cuantificar todas las toxinas en cualquier tipo de muestra y asegurar que cumplen con los límites prescritos en la UE. El problema es que la aparición de nuevas toxinas y la falta de estándares certificados para todas ellas hacen difícil su correcta identificación. Asimismo, una revisión de las biotoxinas marinas reguladas en la legislación de la UE debería ser reconsiderada, ya que algunas toxinas reguladas han demostrado no representar un riesgo para la salud, como es el caso de la YTX y algunas no reguladas han demostrado ser perjudiciales y/o ocurrir en las costas europeas, como las TTXs, CTXs, PlTXs y algunas iminas cíclicas [415]. Por lo tanto, desde que las toxinas de aguas templadas aparecen con más frecuencia en las costas europeas y pueden suponer un problema de salud pública, es necesario establecer límites reglamentarios para estas toxinas.

5. Conclusiones

- La cromatografía líquida acoplada a espectrometría de masas es una técnica eficaz y sensible para separar e identificar, con bajos límites de detección, los análogos de la tetrodotoxina en distintas matrices.

- La tetrodotoxina y el análogo 5,6,11-trideoxyTTX son las toxinas responsables de una intoxicación alimentaria debida al consumo de una caracola de la especie *Charonia lampas* recogida en la costa de Portugal.

- La presencia de tetrodotoxina y sus análogos en gasterópodos y peces recogidos en las costas europeas pone de manifiesto un problema emergente en los sistemas de control que debe de ser considerado.

- El método de cromatografía líquida de alta eficacia con detección fluorescente y oxidación post-columna de Oshima permite determinar la cantidad individual de cada toxina PSP y conocer el perfil de toxinas real de muestras de dinoflagelados.

- El método oficial de cromatografía líquida de alta eficacia con detección fluorescente y oxidación pre-columna de Lawrence es un método sensible que detecta cantidades pequeñas de toxinas PSP, aunque no permite una correcta identificación de todos los análogos presentes en muestras de moluscos.

- Tanto la resina SP700 como el polímero CDP adsorben un amplio rango de toxinas PSP y toxinas lipofílicas; el polímero CDP es más eficaz para la adsorción pasiva de toxinas PSP.

- El uso de la adsorción pasiva, utilizando la resina SP700, puede ser un sistema de alerta temprana de la presencia de toxinas lipofílicas y de las floraciones de microalgas tóxicas en las costas europeas.

6. Bibliografía

1. Bates SS: Ecophysiology and metabolism of ASP toxin production. In *Physiologycal ecology of harmful algal blooms*. Edited by Anderson DM, Cembella AD, Hallegraeff GM. Heildelberg: Springer-Verlag; 1998:405-426.

2. Vargas-Montero M, Freer E: Algal blooms of the toxigenic diatom *Pseudo-Nitzschia* (Bacillariophyceae) in the Golfo de Nicoya, Costa Rica. *Rev Biol Trop* 2004, 52 Suppl 1:127-132.

3. Hallegraeff GM: A review of harmful algal blooms and their apparent global increase. *Phycologia* 1993, 32:79-99.

4. Shimizu Y: Microalgal metabolites: a new perspective. *Annual Revue Microbiol* 1996, 50:431-465.

5. Wright JLC, Cembella AD: Ecophysiology and biosynthesis of polyether marine biotoxins. In *Physiological ecology of harmful algal blooms*. Edited by Anderson DM, Cembella AD, Hallegraeff GM. Heidelberg: Springer-Verlag; 1998:427-452.

6. Baden DG, Trainer VL: Mode of action of toxins of seafood posoning. In *Algal toxins in seafood and drinking water*. Edited by Falconer IA. London: Academic Press; 1993:49-74.

7. Quilliam MA: Chemical methods for lipophilic shellfish toxins. In *Manual on harmful marine microalgae. Volume 11*. Edited by Hallegraeff GM AD, Cembella AD. París: Intergovernmental Oceanographic Commission (UNESCO); 2003:211-246.

8. FAO/IOC/WHO: Food and Agriculture Organization of the United Nations/Intergovernmental Oceanographic Commission of UNESCO/World Health Organization. In *Background document of the Joint FAO/IOC/WHO ad hoc Expert Consultation on Biotoxins in Bivalve Molluscs*. Olso, Norway, September, 26-30; 2004.

9. Wright JLC, Boyd RK, Freitas ASWd, Falk M, Foxall R, Jamieson WD, Laycock MV, McCulloch AW, McInnes AG, Odense P *et al*: Identification of domoic acid, a neuroexcitatory amino acid, in toxic mussels from eastern Prince Edward Island. *Can J Chem Rev Can Chim* 1989, 67(3):481-490.

10. Bates SS, Bird CJ, de Freitas ASW, Foxall RA, Gilgan M, Hanic LA, Johnson GR, McCulloch AW, Odense P, Pocklington R *et al*: Pennate diatom *Nitzschia pungens* as the primary source of domoic acid, a toxin in shellfish from eastern Prince Edward Island, Canada. *Canadian Journal of Fisheries and Aquatic Sciences* 1989, 46(7):1203-1215.

11. Work TM, Beale AM, Fritz L, Quilliam MA, Silver M, Buck K, Wright JLC: Domoic acid intoxication of brown pelicans and cormorants in Santa Cruz, California. In *Toxic phytoplankton blooms in the sea*. Edited by Smayda TJ, Shimizu, Y. . Amsterdam: Elsevier; 1993:643-650.

12. Sierra Beltrán A, Palafox-Uribe M, Grajales-Montiel J, Cruz-Villacorta A, Ochoa JL: Sea bird mortality at Cabo San Lucas, Mexico: evidence that toxic diatom blooms are spreading. *Toxicon* 1997, 35:447-453.

13. Lefebvre KA, Powell CL, Busman M, Doucette GJ, Moeller PDR, Silver JB, Miller PE, Hughes MP, Singaram S, Silver MW *et al*: Detection of domoic acid in northern anchovies and California sea lions associated with an unusual mortality event. *Nat Toxins* 1999, 7:85-92.

14. Sholin CA, Gulland F, Doucette GJ, Benson S, Busman M, Chavez FP, Cordaro J, DeLong R, De Vogelaere A, Harvey J *et al*: Mortality of sea lions along the central California coast liked to a toxic diatom bloom. *Nature* 2000, 403:80-84.

15. Lefebvre KA, Bargu S, Kieckhefer T, Silver MW: From sanddabs to blue whales: the pervasiveness of domoic acid. *Toxicon* 2002, 40(7):971-977.

16. Horner RA, Kusske MB, Moynihan BP, Skinner RN, Wekell JC: Retention of domoic acid by Pacific razor clams, *Siliqua patula* (Dixon, 1789): Preliminary study. *Journal of Shellfish Research* 1993, 12(2):451-456.

17. Miguez A, Fernández LM, Fraga S: First detection of domoic acid in Galicia (NW of Spain). *Harmful and Toxic Algal Blooms* 1996:143-145.

18. Vale P, Sampayo MA: Domoic acid in Portuguese shellfish and fish. *Toxicon* 2001, 39(6):893-904.

19. Amzil Z, Fresnel J, Le Gal D, Billard C: Domoic acid accumulation in French shellfish in relation to toxic species of *Pseudo-nitzschia multiseries* and *P. pseudodelicatissima. Toxicon* 2001, 39(8):1245-1251.

20. Martin JL, Haya K, Burridge LE, Wildish DJ: *Nitzschia pseudodelicatissima* - a source of domoic acid in the Bay of Fundy, eastern Canada. . *Marine Ecology Progress Series* 1990, 67:177-182.

21. Garrison DL, Conrad SM, Eilers PP, Waldron EM: Confirmation of domoic acid production by *Pseudonitzschia australis* (Bacillariophyceae) cultures. *Journal of Phycology* 1992, 28(5):604-607.

22. Lundholm N, Skov J, Pocklington R, Moestrup Ø: Domoic acid, the toxic amino acid responsible for amnesic shellfish poisoning, now in *Pseudo-nitzschia seriata* (Bacillariophyceae) in Europe. *Phycologia* 1994, 33(6):475-478.

23. Wright JLC, Falk M, McInnes AG, Walter JA: Identification of isodomoic acid D and two new geometrical isomers of domoic acid in toxic mussels. *Can J Chem* 1990, 68:22-25.

24. Doucette TA, Tasker RA: Domoic Acid: Detection Methods, Pharmacology, and Toxicology. In *Seafood and Freshwater Toxins: Pharmacology, Physiology, and Detection*. Edited by Botana LM. Boca Raton-New York: Taylor & Francis; 2008:398-422.

25. Bettler B, Mulle C: Review: Neurotransmitter receptors II . AMPA and kainate receptors *Neuropharmacology* 1995, 34(2):123-139.

26. Olney JW, Fuller T, Gubareff T: Acute dendrotoxic changes in the hippocampus of kainate treated rats. *Brain Res* 1979, 176(1):91-100.

27. Jeffery B, Barlow T, Moizer K, Paul S, Boyle C: Amnesic shellfish poison. *Food Chem Toxicol* 2004, 42(4):545-557.
28. Perl TM, Bedard LM, Kosatsky TMD, Hockin JC, Todd ECD, Remis RS: An outbreak of toxic encephalopathy caused by eating mussels contaminated with domoic acid. *New Engl J Med* 1990, 322:1775-1780.

29. Teitelbaum JS, Zatorre RJ, Carpenter S, Gendron D, Evans AC, Gjedde A, Cashman NR: Neurologic sequelae of domoic acid intoxication due to the ingestion of contaminated mussels. *N Engl J Med* 1990 322(25):1781-1787.

30. Lawrence JF, Charbonneau CF, Ménard C, Quilliam MA, Sim PG: Liquid chromatographic determination of domoic acid in shellfish products using the paralytic shellfish poison extraction procedure of the association of official analytical chemists. *Journal of Chromatography A* 1989, 462:349-356.

31. Quilliam MA, Sim PG, McCulloch AW, McInnes AG: High-Performance Liquid Chromatography of Domoic Acid, a Marine Neurotoxin, with Application to Shellfish and Plankton. *International Journal of Environmental Analytical Chemistry* 1989, 36(3):139 - 154.

32. Lawrence JF, Charbonneau CF, Ménard C: Liquid chromatographic determination of domoic acid in mussels, using AOAC paralytic shellfish poison extraction procedure: collaborative study. *J Assoc Off Anal Chem* 1991, 74(1):68-72.

33. AOAC: Official Method 991.26. Domoic acid in mussels, liquid chromatography method. In *Official Methods of Analysis of AOAC International*. Edited by Horowitz W. Gaithersburg, Maryland, USA: AOAC International; 2000.

34. (CEN) CE: Foodstuffs- Determination of Domoic Acid in Mussels by HPLC. *European Committee for Standardization (CEN)* 2003.

35. AOAC: Official Method 2006.02. In *Official Methods of Analysis of AOAC International*. 19th Ed., AOAC International edition. Gaithersburg, MD USA; 2006.

36. EFSA: Marine biotoxins in shellfish-Domoic acid. Panel on Contaminants in the Food Chain (CONTAM) *European Food Safety Authority Journal* 2009, 1181 1-61.

37. McNabb P, Selwood AI, Holland PT: Multiresidue Method for Determination of Algal Toxins in Shellfish: Single-Laboratory Validation and Interlaboratory Study. *J Assoc Off Anal Chem* 2005, 88(3):761.

38. Zhao JY, Thibault P, Quilliam MA: Analysis of domoic acid isomers in seafood by capillary electrophoresis. *Electrophoresis* 1997, 18(2):268-276.

39. Roeder K, Erler K, Kibler S, Tester P, Van The H, Nguyen-Ngoc L, Gerdts G, Luckas B: Characteristic profiles of Ciguatera toxins in different strains of *Gambierdiscus spp*. *Toxicon* 2010, 56(5):731-738.

40. Lehane L, Lewis RJ: Ciguatera: recent advances but the risk remains. *Int J Food Microbiol* 2000, 61(2-3):91-125.

41. Hamilton B, Hurbungs M, Vernoux JP, Jones A, Lewis RJ: Isolation and characterisation of Indian Ocean ciguatoxin. *Toxicon* 2002, 40:685-693.

42. Dickey RW, Plakas SM: Ciguatera: a public health perspective. *Toxicon* 2010, 56(2):123-136.

43. Bentur Y, Spanier E: Ciguatoxin-like substances in edible fish on the eastern Mediterranean. *Clinical Toxicology* 2007, 45(6):695-700.

44. Aligizaki K, Nikolaidis G: Morphological identification of two tropical dinoflagellates of the genera *Gambierdiscus* and *Sinophysis* in the Mediterranean Sea. *Journal of Biology Research* 2008, 9:75-82.

45. Perez-Arellano JL, Luzardo OP, Perez Brito A, Cabrera Hernandez C, Zumbado M, Carranza C, Angel-Moreno A, Dickey RW, Boada LD: First report of ciguatera fish poisoning in West-African coast: a public health concern in the Canary Islands. *Emerging Infectious Diseases* 2005, 11:1981-1982.

46. Otero P, Perez S, Alfonso A, Vale C, Rodriguez P, Gouveia NN, Gouveia N, Delgado J, Vale P, Hirama M *et al*: First toxin profile of ciguateric fish in Madeira Arquipelago (Europe). *Anal Chem* 2010, 82(14):6032-6039.

47. Dechraoui MY, Wacksman JJ, Ramsdell JS: Species selective resistance of cardiac muscle voltage gated sodium channels: characterization of brevetoxin and ciguatoxin binding sites in rats and fish. *Toxicon* 2006, 48(6):702-712.

48. Nguyen-Huu TD, Mattei C, Wen PJ, Bourdelais AJ, Lewis RJ, Benoit E, Baden DG, Molgo J, Meunier FA: Ciguatoxin-induced catecholamine secretion in bovine chromaffin cells: mechanism of action and reversible inhibition by brevenal. *Toxicon* 2010, 56(5):792-796.

49. Friedman MA, Fleming LE, Fernandez M, Bienfang P, Schrank K, Dickey R, Bottein MY, Backer L, Ayyar R, Weisman R *et al*: Ciguatera fish poisoning: treatment, prevention and management. *Mar Drugs* 2008, 6(3):456-479.

50. UE: Reglamento (CE) Nº 854/2004 Del Parlamento Europeo y del Consejo de 29 de abril de 2004 por el que se establecen normas específicas para la organización de controles oficiales de los productos de origen animal destinados al consumo humano. *Diario Oficial de la Unión Europea* 2004, L 139:206.

51. EFSA: Scientific Opinion on marine biotoxins in shellfish – Emerging toxins: Ciguatoxin group. Panel on Contaminants in the Food Chain (CONTAM). *European Food Safety Authority Journal* 2010, 8(6):1627.

52. Lewis RJ, Sellin M: Recovery of ciguatoxin from fish flesh. *Toxicon* 1993, 31(10):1333-1336

53. Campora CE, Dierking J, Tamaru CS, Hokama Y, Vincent D: Detection of ciguatoxin in fish tissue using sandwich ELISA and neuroblastoma cell bioassay. *Journal of Clinical Laboratory Analysis* 2008, 22(4):246-253.

54. Manger RL, Leja LS, Lee SY, Hungerford JM, Hokama Y, Dickey RW, Granade HR, Lewis RJ, Yasumoto T, Wekell MM: Detection of sodium channel toxins:

directed cytotoxicity assays of purified ciguatoxins, brevetoxins, saxitoxins, and seafood extracts. *J AOAC* 1995, 78:521-527.

55. Perez S, Vale C, Alonso E, Alfonso C, Rodriguez P, Otero P, Alfonso A, Vale P, Hirama M, Vieytes MR *et al*: A comparative study of the effect of ciguatoxins on voltage-dependent Na+ and K+ channels in cerebellar neurons. *Chem Res Toxicol* 2011, 24(4):587-596.

56. Lewis RJ, Yang A, Jones A: Rapid extraction combined with LC-tandem mass spectrometry (CREM-LC/MS/MS) for the determination of ciguatoxins in ciguateric fish flesh. *Toxicon* 2009, 54(1):62-66.

57. Pottier I, Vernoux JP, Jones A, Lewis RJ: Analysis of toxin profiles in three different fish species causing ciguatera fish poisoning in Guadeloupe, French West Indies. *Food Addit Contam* 2002, 19(11):1034-1042.

58. Moore RE, Scheuer PJ: Palytoxin: a new marine toxin from a coelenterate. *Science* 1971, 172:495-498.

59. Uemura D, Hirata Y, Iwashita T, Naoki H: Studies on Palytoxins. *Tetrahedron* 1985, 41:1007-1017.

60. Oku N, Sata NU, Matsunaga S, Uchida H, Fusetani N: Identification of palytoxin as a principle which causes morphological changes in rat 3Y1 cells in the zoanthid *Palythoa* aff. *margaritae*. *Toxicon* 2004, 43:21-25.

61. Ciminiello P, Dell'Aversano C, Dello Iacovo E, Fattorusso E, Forino M, Grauso L, Tartaglione L, Florio C, Lorenzon P, De Bortoli M *et al*: Stereostructure and biological activity of 42-hydroxy-palytoxin: a new palytoxin analogue from Hawaiian *Palythoa* subspecies. *Chem Res Toxicol* 2009, 22:1851-1859.

62. Usami M, Satake M, Ishida S, Inoue A, Kan Y, Yasumoto T: Palytoxin analogs from the dinoflagellate *Ostreopsis siamensis. J Am Chem Soc* 1995, 117(5389-5390).

63. Lenoir S, Ten-Hage L, Turquet J, Quod JP, Bernard C, Hennion MC: First evidence of palytoxin analogues from an *Ostreopsis mascarenensis* (Dinophyceae) benthic bloom in southwestern Indian Ocean. *J Phycol* 2004, 40:1042-1051.

64. Rossi R, Castellano V, Scalco E, Serpe L, Zingone A, Soprano V: New palytoxin-like molecules in Mediterranean *Ostreopsis* cf. *ovata* (dinoflagellates) and in *Palythoa tuberculosa* detected by liquid chromatography-electrospray ionization time-of-flight mass spectrometry. *Toxicon* 2010, 56:1381-1387.

65. Aligizaki K, Nikolaidis G: The presence of the potentially toxic genera *Ostreopsis* and *Coolia* (Dinophyceae) in the North Aegean Sea, Greece. *Harmful Algae* 2006, 5(6):717-730.

66. Aligizaki K, Katikou P, Nikolaidis G, Panou A: First episode of shellfish contamination by palytoxin-like compounds from *Ostreopsis* species (Aegean Sea, Greece). *Toxicon* 2008, 51:418-427.

67. Aligizaki K, Katikou P, Milandri A, Diogene J: Occurrence of palytoxin-group toxins in seafood and future strategies to complement the present state of the art. *Toxicon* 2011, 57:390-399.

68. Sansoni G, Borghini B, Camici G, Casotti M, Righini P, Rustighi C: Fioriture algali di *Ostreopsis ovata* (Gonyaulacales: Dinophyceae): un problema emergente. *Biología Ambiental* 2003, 17:17-23.

69. Vila M, Garcés E, Masó M: Potentially toxic epiphytic dinoflagellate assemblages on macroalgae in the NW Mediterranean. *Aquatic Microbial Ecology* 2001, 26:51-60.

70. Ciminiello P, Dell'aversano C, Dello Iacovo E, Fattorusso E, Forino M, Tartaglione L, Rossi R, Soprano V, Capozzo D, Serpe L: Palytoxin in seafood by liquid chromatography tandem mass spectrometry: investigation of extraction efficiency and matrix effect. *Anal Bioanal Chem* 2011.

71. Tan CH, Lau CO: Palytoxin: chemistry and detection. In *Seafood and Freshwater Toxins: Pharmacology, Physiology and Detection*. Edited by Botana LM. New York: Marcel Dekker Inc.; 2000:533.

72. Bellocci M, Sala GL, Prandi S: The cytolytic and cytotoxic activities of palytoxin. *Toxicon* 2011, 57(3):449-459.

73. Ciminiello P, Dell'Aversano C, Dello Iacovo E, Fattorusso E, Forino M, Tartaglione L: LC-MS of palytoxin and its analogues: State of the art and future perspectives. *Toxicon* 2011, 57(3):376-389.

74. Munday R: Palytoxin toxicology: animal studies. *Toxicon* 2011, 57(3):470-477.

75. Habermann E: Review article. Palytoxin acts through Na+/K+ ATPase. *Toxicon* 1989, 27(1175-1187).

76. Wu CH: Palytoxin: membrane mechanism of action. *Toxicon* 2009, 54:1183-1189.

77. Rossini GP, Bigiani A: Palytoxin action on the Na+, K+-ATPase and the disruption of ion equilibria in biological systems. *Toxicon* 2011, 57(3):429-439.

78. Artigas P, Gadsby DC: Na+/K+ -pump ligands modulate gating of palytoxin-induced ion channels. *PNAS* 2003, 100(501-505).

79. Deeds JR, Schwartz MD: Human risk associated with palytoxin exposure. *Toxicon* 2010, 56(2):150-162.

80. Tubaro A, Durando P, Del Favero G, Ansaldi F, Icardi G, Deeds JR, Sosa S: Case definitions for human poisonings postulated to palytoxins exposure. *Toxicon* 2011, 57(3):478-495.

81. EFSA: Scientific Opinion on marine biotoxins in shellfish – Palytoxin group. Panel on Contaminants in the Food Chain (CONTAM). *European Food Safety Authority Journal* 2009, 7(12):1393.

82. Levine L, Fujiki H, Gjika HB, Vanvunakis H: Production of antibodies to palytoxin-neutralization of several biological properties of palytoxin. *Toxicon* 1987, 25(12):1273-1282.

83. Bignami GS, Raybould TJG, Sachinvala ND, Grothaus PG, Simpson SB, Lazo CB, Byrnes JB, Moore RE, Vann DC: Monoclonal antibody-based enzyme-linked immunoassays for the measurement of palytoxin in biological samples. *Toxicon* 1992, 30(7):687-700.

84. Garet E, Cabado AG, Vieites JM, Gonzalez-Fernandez A: Rapid isolation of single-chain antibodies by phage display technology directed against one of the most potent marine toxins: Palytoxin. *Toxicon* 2010, 55(8):1519-1526.

85. Bignami GS: A rapid and sensitive hemolysis neutralization assay for palytoxin. *Toxicon* 1993, 31(6):817-820.

86. Yakes BJ, Degrasse SL, Poli M, Deeds JR: Antibody characterization and immunoassays for palytoxin using an SPR biosensor. *Anal Bioanal Chem* 2011, 400(9):2865-2869.

87. Riobo P, Franco JM: Palytoxins: biological and chemical determination. *Toxicon* 2011, 57(3):368-375.

88. Aune T, Yndestad M: Diarrhetic shellfish poisoning, chap. 5. In *Algal Toxins in Seafood and Drinking Water*. Edited by Falconer IR. London: Academic Press; 1993.

89. Yasumoto T, Oshima Y, Yamaguchi M: Ocurrence of a new type of shellfish poisoning in the Tohoku district. *Bulletin of the Japanese Society of Scientific Fisheries* 1978, 44(11):1249-1255.

90. Yasumoto T, Oshima Y, Sugawara W, Fukuyo Y, Oguri H, Igarashi T, Fujita N: Identification of *Dinophysis fortii* as the causative organism of diarrhetic shellfish poisoning in the Tohoku district. *B Jpn Soc Sci Fish* 1980, 46:1405-1411.

91. Yasumoto T: Diarrhetic shellfish toxin and okadai acid. *Jikken Igaku* 1990, 8:1243.

92. Tubaro A, Sosa S, Bornancin A, Hungerford J: Pharmacology and Toxicology of Diarrheic Shellfish Toxins. In *Seafood and Freshwater Toxins: Pharmacology, Physiology, and Detection, 2nd edition*. Edited by Botana LM. Boca Raton, FL: Taylor and Francis Group; 2008:229-253.

93. Hallegraeff GM: Harmful algal blooms: a global overview. In *Manual on Harmful Marine Microalgae. Volume 25*. Edited by Hallegraeff GM, Anderson DM, Cembella AD. Saint-Berthevin: UNESCO; 2003.

94. Tachibana K, Scheuer PJ, Tsukitani Y, Kikuchi H, Van Engen D, Clardy J, Gopichand Y, Schmitz FJ: Okadaic acid, a cytotoxic polyether from two marine sponges of the genus *Halichondria*. *J Am Chem Soc* 1981, 103(9):2469–2471.

95. Lee JS, Igarashi TT, Fraga S, Dahl E, Hovgaard P, Yasumoto T: Determination of diarrhetic shellfish toxins in various dinoflagellate species. *J Appl Phycol* 1989, 1:147-152.

96. Fernández ML, Reguera B, Ramilo I, Martízez A: Toxin content of *Dinophysis acuminata*, *D. acuta* and *D. caudata* from the Galician Rías Bajas. In *Harmful Algal Blooms*. Edited by Hallegraeff GM, Blackburn SI, Bolch CJ, Lewis RJ. Paris: Intergovernmental Oceanographic Commision of UNESCO; 2001:360-363.

97. Morton SL, Moeller PDR, Young KA, Lanoue B: Okadaic acid production from the marine dinoflagellate *Prorocentrum belizaneum* Faust isolated from the Belizean coral reef ecosystem. *Toxicon* 1998, 36(1):201-206.

98. Ten-Hage L, Delaunay N, Pichon V, Couté A, Puiseux-Dao S, Turquet J: Okadaic acid production from the marine benthic dinoflagellate *Prorocentrum arenarium* Faust (Dinophyceae) isolated from Europa Island coral reef system (SW Indian Ocean). *Toxicon* 2000, 38:1043-1054.

99. Vale P: Differential dynamics of dinophysistoxins and pectenotoxins, part II: Offshore bivalve species *Toxicon* 2006, 47(2):163-173.

100. Vale P, Sampayo MAM: First confirmation of human diarrhoeic poisonings by okadaic acid esters after ingestion of razor clams (*Solen marginatus*) and green crabs (*Carcinus maenas*) in Aveiro lagoon, Portugal and detection of okadaic acid esters in phytoplankton. *Toxicon* 2002, 40(7):989-996.

101. Murata M, Shimatani M, Sugitani H, Oshima Y, Yasumoto T: Isolation and structural elucidation of the causative toxin of the diarrhetic shellfish poisoning. *Bull Japn Soc Sci Fish* 1982, 48:549-552.

102. Marr JC, Hu T, Pleasance S, Quilliam MA, Wright JLC: Detection of new 7-O-acyl derivatives of diarrhetic shellfish poisoning toxins by liquid chromatography-mass spectrometrystar. *Toxicon* 1992, 30(12):1621-1630.

103. Takai A, Murata M, Torigoe K, Isobe M, Mieskes G, Yasumoto T: Inhibitory effect of okadaic acid derivatives on protein phosphatases. A study on structure-affinity relationship. *Biochemical Journal* 1992, 284:539-544.

104. Dounay AB, Forsyth CJ: Okadaic acid: the archetypal serine/threonine protein phosphatase inhibitor. *Current Medicinal Chemistry* 2002, 9(22):1939-1980.

105. Hamano Y, Kinoshita Y, Yasumoto T: Enteropathogenity of diarrhetic shellfish toxins in intestinal models. *J Food Hyg Soc Jpn* 1986, 27:375-379.

106. Cohen P, Holmes CFB, Tsukitani Y: Okadaic acid: a new probe for the study of cellular regulation *Trends in Biochemical Sciences* 1990, 15(3):98-102.

107. Van Egmond HP, Aune T, Lassus P, Speijers G, Waldock M: Paralytic and diarrhoeic shellfish poisons: occurrence in Europe, toxicity, analysis and regulation. *J Nat Toxins* 1993, 2:41-83.

108. Hallegraeff GM: Harmful Algal Blooms: A global overview. In *Manual on Harmful Marine Microalgae*. Edited by Hallegraeff GM, Anderson DM, Cembella AD: IOC Manuals and Guides No. 33. UNESCO; 1995:1-22.

109. EU: Commission Regulation (EC) No 853/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific hygiene rules for food of animal origin. *Off J Eur Union* 2004, L139(55-255).

110. EFSA: Marine biotoxins in shellfish – okadaic acid and analogues. Scientific Opinion of the Panel on Contaminants in the Food chain. *European Food Safety Authority Journal* 2008, 589:1-62.

111. EU: Commission Regulation (EC) No 2074/2005 of 5 December 2005 laying down implementing measures for certain products under Regulation (EC) No 853/2004 of the European Parliament and of the Council and for the organisation of official controls under Regulation (EC) No 854/2004 of the European Parliament and of the Council and Regulation (EC) No 882/2004 of the European Parliament and of the Council, derogating from Regulation (EC) No 852/2004 of the European Parliament and of the Council and amending Regulations (EC) No 853/2004 and (EC) No 854/2004. *Off J Eur Union* 2005, L338:27.

112. EU: Commission Regulation (EU) No 15/2011 of 10 January 2011 amending Regulation (EC) No 2074/2005 as regards recognised testing methods for detecting marine biotoxins in live bivalve molluscs. *Official Journal of the European Union* 2011, L6:3.

113. McNabb P, Selwood AI, Holland PT, Aasen J, Aune T, Eaglesham G, Hess P, Igarishi M, Quilliam M, Slattery D *et al*: Multiresidue method for determination of algal toxins in shellfish: single-laboratory validation and interlaboratory study. *J AOAC Int* 2005, 88(3):761-772.

114. These A, Klemm C, Nausch I, Uhlig S: Results of a European interlaboratory method validation study for the quantitative determination of lipophilic marine biotoxins in raw and cooked shellfish based on high-performance liquid chromatography-tandem mass spectrometry. Part I: collaborative study. *Anal Bioanal Chem* 2011, 399(3):1245-1256.

115. Otero P, Alfonso A, Alfonso C, Rodriguez P, Vieytes MR, Botana LM: Effect of Uncontrolled Factors in a Validated Liquid Chromatography-Tandem Mass Spectrometry Method Question Its Use As a Reference Method for Marine Toxins: Major Causes for Concern. *Anal Chem* 2011.

116. Madigan TL, Lee KG, Padula DJ, McNabb P, Pointon AM: Diarrhetic shellfish poisoning (DSP) toxins in South Australian shellfish. *Harmful Algae* 2006, 5(2):119-123.

117. Pavela-Vrancic M, Mestrović V, Marasović I, Gillman M, Furey A, James KJ: DSP toxin profile in the coastal waters of the central Adriatic Sea. *Toxicon* 2002, 40(11):1601-1607.

118. Pavela-Vrancic M, Mestrović V, Marasović I, Gillman M, Furey A, James KK: The occurrence of 7-epi-pectenotoxin-2 seco acid in the coastal waters of the central Adriatic (Kastela Bay). *Toxicon* 2001, 39(6):771-779.

119. Puente PF, Sáez MJ, Hamilton B, Furey A, James KJ: Studies of polyether toxins in the marine phytoplankton, *Dinophysis acuta*, in Ireland using multiple tandem mass spectrometry. *Toxicon* 2004, 44(8):919-926.

120. Draisci R, Palleschi L, Giannetti L, Lucentini L, James KJ, Bishop AG, Satake M, Yasumoto T: New approach to the direct detection of known and new

diarrhoeic shellfish toxins in mussels and phytoplankton by liquid chromatography- mass spectrometry. *J Chromatogr A* 1999, 847(1-2):213-221.

121. Sasaki K, Wright JLC, Yasumoto T: Identification and Characterization of Pectenotoxin (PTX) 4 and PTX7 as Spiroketal Stereoisomers of Two Previously Reported Pectenotoxins. *J Org Chem* 1998, 63(8):2475–2480.

122. MacKenzie L, Holland P, McNabb P, Beuzenberg V, Selwood A, Suzuki T: Complex toxin profiles in phytoplankton and Greenshell mussels (*Perna canaliculus*), revealed by LC–MS/MS analysis. *Toxicon* 2002, 40(9):1321-1330.

123. Puente PF, Sáez MJF, Hamilton B, Lehane M, Ramstad H, Furey A, James KJ: Rapid determination of polyether marine toxins using liquid chromatography–multiple tandem mass spectrometry. *Journal of Chromatography A* 2004, 1056(1-2):77-82.

124. Vale P, Sampayo MAM: Pectenotoxin-2 seco acid, 7-epi-pectenotoxin-2 seco acid and pectenotoxin-2 in shellfish and plankton from Portugal. *toxicon* 2002, 40:979-987.

125. Fernández ML, Reguera B, González-Gil S, Míguez A: Pectenotoxin-2 in single-cell isolates of *Dinophysis caudata* and *Dinophysis acuta* from the Galician Rías (NW Spain) *Toxicon* 2006, 48(5):477-490.

126. Vershinin A, Moruchkov A, Morton S, Leighfield TA, Quilliam MA, Ramsdell JS: Phytoplankton composition of the Kandalaksha Gulf, Russian White Sea: Dinophysis and lipophilic toxins in the blue mussel (*Mytilus edulis*) *Harmful Algae* 2006, 5(5):558-564.

127. Stobo LA, Lacaze JP, Scott AC, Gallacher S, Smith EA, Quilliam MA: Liquid chromatography with mass spectrometry-detection of lipophilic shellfish toxins. *J AOAC Int* 2005, 88(5):1371-1382.

128. Draisci R, Lucentini L, Giannetti L, Boria P, Poletti R: First report of pectenotoxin-2 (PTX-2) in algae (*Dinophysis fortii*) related to seafood poisoning in Europe *Toxicon* 1996, 34(8):923-935.

129. Fernández ML, Miguez A, Moroño A, Arévalo F, Pazos Y, Salgado C, Correa J, Blanco J, González-Gil S, Reguera B: First report of pectenotoxin-2 in phytoplankton net-hauls and mussels from the Galician Rías Baixas during proliferations of *Dinophysis acuta* and *D. caudata.* In *Molluscan Shellfish Safety* Edited by Villalba A, Reguera B, Romalde J, Beiras R. Santiago de Compostela, Spain. Consellería de Pesca e Asuntos Marítimos da Xunta de Galicia and Intergovernmental Oceanographic Commission of UNESCO.; 2003:75–83.

130. MacKenzie L, Beuzenberg V, Holland P, McNabb P, Suzuki T, Selwood A: Pectenotoxin and okadaic acid-based toxin profiles in *Dinophysis acuta* and *Dinophysis acuminata* from New Zealand. *Harmful Algae* 2005, 4:75-85.

131. Yasumoto T, Murata M, Oshima Y, Sano M, Matsumoto GK, Clardy J: Diarrhetic shellfish toxins. *Tetrahedron* 1985, 41:1019.

132. Yasumoto T, Murata M: Marine toxins. *Chem Rev* 1993, 93:1897.

133. Yasumoto T, Murata M, Lee JS, Torigoe K: Polyether toxins produced by dinoflagellates. In *Proc Mycotoxins and Phycotoxins '88*. Edited by Natori S, Hashimoto K, Ueno Y. Amsterdam: Elsevier; 1989:375.
134. Suzuki T: Chemistry, Metabolism, and Chemical Detection Methods of Pectenotoxins. In *Seafood and Freshwater Toxins: Pharmacology, Physiology, and Detection*. 2nd edition. Edited by Botana LM. Boca Raton, FL: CRC Press. Taylor & Francis Group; 2008:343-380.

135. Yasumoto T, Murata M, Oshima Y, Matsumoto GK, Clardy J: Diarrhetic shellfish poisoning In *ACS Symposium Series No 262, Seafood Toxins*. Edited by Ragelis EP. Washington, D.C.: ACS; 1984:207.

136. Suzuki T, Mitsuya T, Matsubara H, Yamasaki M: Determination of pectenotoxin-2 after solid-phase extraction from seawater and from the dinoflagellate *Dinophysis fortii* by liquid chromatography with electrospray mass spectrometry and ultraviolet detection: Evidence of oxidation of pectenotoxin-2 to pectenotoxin-6 in scallops. *Journal of Chromatography A* 1998, 815(1):155-160.

137. EFSA: Marine biotoxins in shellfish – Pectenotoxin group. Scientific Opinion of the Panel on Contaminants in the Food chain. *European Food Safety Authority Journal* 2009, 1109:1-47.

138. Satake M, MacKenzie L, Yasumoto T: Identification of *Protoceratium reticulatum* as the biogenetic origin of yessotoxin. *Natural toxins* 1997, 5(4):164-167.

139. Paz B, Riobo P, Fernandez ML, Fraga S, Franco JM: Production and release of yessotoxins by the dinoflagellates *Protoceratium reticulatum* and *Lingulodinium polyedrum* in culture. *Toxicon* 2004, 44(3):251-258.

140. Rhodes L, McNabb P, de Salas M, Briggs L, Beuzenberg V, Gladstone M: Yessotoxin production by *Gonyaulax spinifera. Harmful Algae* 2006, 5(2):148-155.

141. Murata M, Kumagai M, Lee JS, Yasumoto T: Isolation and structure of yessotoxin, a novel polyether compound implicated in diarrhetic shellfish poisoning. *Tetrahedron Letters* 1987, 28(47):5869-5872

142. Lee J-S, Tangen K, Dahl E, Hovgaard P, Yasumoto T: Diarrhetic Shellfish Toxins In Norwegian Mussels. *Nippon Suisan Gakkaishi* 1988, 54(11):1953-1957.

143. Yasumoto T, Takizawa A: Fluorometric measurement of yessotoxins in shellfish by high-pressure liquid chromatography. *Biosci Biotechnol Biochem* 1997, 61(10):1775-1777.

144. Finch SC, Wilkins AL, Hawkes AD, Jensen DJ, MacKenzie AL, Beuzenberg V, Quilliam MA, Olseng CD, Samdal IA, Aasen J *et al*: Isolation and identification of (44-R,S)-44,55-dihydroxyyessotoxin from *Protoceratium reticulatum*, and its occurrence in extracts of shellfish from New Zealand, Norway and Canada. *Toxicon* 2005, 46(2):160-170.

145. Ciminiello P, Fattorusso E, Forino M, Magno S, Poletti R, Satake M, Viviani R, Yasumoto T: Yessotoxin in mussels of the northern Adriatic Sea. *Toxicon* 1997, 35(2):177-183.

146. Ciminiello P, Fattorusso E: Chemistry, Metabolism, and Chemical Analysis. In *Seafood and Freshwater Toxins: Pharmacology, Physiology, and Detection, 2nd edition*. Edited by Botana LM. Boca Raton, FL: Taylor and Francis; 2008:287-313.

147. Paz B, Daranas AH, Norte M, Riobo P, Franco JM, Fernandez JJ: Yessotoxins, a group of marine polyether toxins: an overview. *Mar Drugs* 2008, 6(2):73-102.

148. Ogino H, Kumagai M, Yasumoto T: Toxicologic evaluation of Yessotoxin. *Natural Toxins* 1997, 5(6):255-259.

149. Terao K, Ito E, Oarada M, Murata M, Yasumoto T: Histopathological studies on experimental marine toxin poisoning-5. The effects in mice of yessotoxin isolated from *Patinopecten yessoensis* and of a desulfated derivative. *Toxicon* 1990, 28(9):1095-1104.

150. Tubaro A, Sosa S, Carbonatto M, Altinier G, Vita F, Melato M, Satake M, Yasumoto T: Oral and intraperitoneal acute toxicity studies of yessotoxin and homoyessotoxins in mice. *Toxicon* 2003, 41(7):783-792.

151. Tubaro A, Sosa S, Altinier G, Soranzo MR, Satake M, Della Loggia R, Yasumoto T: Short-term oral toxicity of homoyessotoxins, yessotoxin and okadaic acid in mice. *Toxicon* 2004, 43(4):439-445.

152. Suárez-Korsnes M, Hetland DL, Espenes A, Tranulis MA, Aune T: Apoptotic events induced by yessotoxin in myoblast cell lines from rat and mouse. *Toxicol in Vitro* 2006, 20:1077-1087.

153. de la Rosa LA, Alfonso A, Vilarino N, Vieytes MR, Yasumoto T, Botana LM: Maitotoxin-induced calcium entry in human lymphocytes: modulation by yessotoxin, Ca(2+) channel blockers and kinases. *Cell Signal* 2001, 13(10):711-716.

154. de la Rosa LA, Alfonso A, Vilariño N, Vieytes MR, Botana LM: Modulation of cytosolic calcium levels of human lymphocytes by yessotoxin, a novel marine phycotoxin. *Biochem Pharmacol* 2001, 61:827-833.

155. Alfonso A, de la Rosa LA, Vieytes MR, Yasumoto T, Botana LM: Yessotoxin, a novel phycotoxin, activates phosphodiesterase activity. Effect of yessotoxin on cAMP levels in human lymphocytes. *Biochem Pharmacol* 2003, 65:193-208.

156. Pazos M-J, Alfonso A, Vieytes MR, Yasumoto T, Botana LM: Study of the Interaction between Different Phosphodiesterases and Yessotoxin Using a Resonant Mirror Biosensor. *Chem Res Toxicol* 2006, 19:794-800.

157. Alfonso A, Alfonso C: Pharmacology and Mechanism of Action: Biological Detection. In *Seafood and Freshwater Toxins: Pharmacology, Physiology, and Detection, 2nd edition*. Edited by Botana LM. Boca Raton, FL: Taylor and Francis Group; 2008:315-327.

158. Leira F, Alvarez C, Vieites JM, Vieytes MR, Botana LM: Characterization of distinct apoptotic changes induced by okadaic acid and yessotoxin in the BE(2)-M17 neuroblastoma cell line. *Toxinol in Vitro* 2002, 16:23-31.

159. Malaguti C, Ciminiello P, Fattorusso E, Rossini GP: Caspase activation and death induced by yessotoxin in HeLa cells. *Toxicol in Vitro* 2002, 16:357-363.

160. Suárez-Korsnes M, Hetland DL, Espenes A, Aune T: Induction of apoptosis by YTX in myoblast cell lines via mitochondrial signalling transduction pathway. . *Toxicol in Vitro* 2006, 20:1419-1426.

161. Ronzitti G, Callegari F, Malaguti C, Rossini GP: Selective disruption of the E-cadherin-catenin system by an algal toxin. . *Br J Cancer* 2004, 90:1100-1107.

162. EU: Commission Decision 2002/225/EC of 15 March 2002 laying down detailed rules for the implementation of Council Directive 91/492/EEC as regards the maximum levels and the methods of analysis of certain marine biotoxins in bivalve molluscs, echinoderms, tunicates and marine gastropods. *Official Journal of the European Communities* 2002, L75(62-63).

163. EFSA: Marine biotoxins in shellfish – Yessotoxin group. Scientific Opinion of the Panel on Contaminants in the Food chain. *European Food Safety Authority Journal* 2008, 907:1-62.

164. Gago-Martínez A, Leão JM, Piñeiro N, Carballal E, Vaquero E, Nogueiras M, Rodríguez-Vázquez JA: An Application of Capillary Electrophoresis for the Analysis of Algal Toxins from the Aquatic Environment. *International Journal of Environmental Analytical Chemistry* 2003, 83(6): 443 - 456.

165. Alfonso C, Alfonso A, Vieytes MR, Yasumoto T, Botana LM: Quantification of yessotoxin using the fluorescence polarization technique and study of the adequate extraction procedure. *Anal Biochem* 2005, 344(2):266-274.

166. Briggs LR, Miles CO, Fitzgerald JM, Ross KM, Garthwaite I, Towers NR: Enzyme-linked immunosorbent assay for the detection of yessotoxin and its analogues. *J Agric Food Chem* 2004, 52(19):5836-5842.

167. Pazos MJ, Alfonso A, Vieytes MR, Yasumoto T, Botana LM: Kinetic analysis of the interaction between yessotoxin and analogues and immobilized phosphodiesterases using a resonant mirror optical biosensor. *Chem Res Toxicol* 2005, 18(7):1155-1160.

168. Fonfria ES, Vilarino N, Vieytes MR, Yasumoto T, Botana LM: Feasibility of using a surface plasmon resonance-based biosensor to detect and quantify yessotoxin. *Anal Chim Acta* 2008, 617(1-2):167-170.

169. McMahon T, Silke J: Winter toxicity of unknown aetiology in mussels. *Harmful Algae News* 1996, 14:2.

170. Ito E, Satake M, Ofuji K, Kurita N, McMahon T, James K, Yasumoto T: Multiple organ damage caused by a new toxin azaspiracid, isolated from mussels produced in Ireland. *Toxicon* 2000, 38(7):917-930.

171. Ito E, Satake M, Ofuji K, Higashi M, Harigaya K, McMahon T, Yasumoto T: Chronic effects in mice caused by oral administration of sublethal doses of azaspiracid, a new marine toxin isolated from mussels. *Toxicon* 2002, 40(2):193-203.

172. Satake M, Ofuji K, Naoki H, James KJ, Furey A, McMahon T, Silke J, Yasumoto T: Azaspiracid, a new marine toxin having unique spiro ring assemblies, isolated from Irish mussels, *Mytilus edulis*. *J Am Chem Soc* 1998, 120:9967-9968.

173. Nicolaou KC, Chen DY, Li Y, Uesaka N, Petrovic G, Koftis TV, Bernal F, Frederick MO, Govindasamy M, Ling T *et al*: Total synthesis and structural elucidation of azaspiracid-1. Synthesis-based analysis of originally proposed structures and indication of their non-identity to the natural product. *J Am Chem Soc* 2006, 128(7):2258-2267.

174. Nicolaou KC, Koftis TV, Vyskocil S, Petrovic G, Tang W, Frederick MO, Chen DY, Li Y, Ling T, Yamada YM: Total synthesis and structural elucidation of azaspiracid-1. Final assignment and total synthesis of the correct structure of azaspiracid-1. *J Am Chem Soc* 2006, 128(9):2859-2872.

175. McMahon T, Silke J: Re-occurrence of winter toxicity. *Harmful Algae News* 1998, 17:12.

176. Ofuji K, Satake M, McMahon T, Silke J, James KJ, Naoki H, Oshima Y, Yasumoto T: Two analogs of azaspiracid isolated from mussels, *Mytilus edulis*, involved in human intoxication in Ireland. *Natural Toxins* 1999, 7(3):99-102.

177. Nicolaou KC, Frederick MO, Petrovic G, Cole KP, Loizidou EZ: Total Synthesis and Confirmation of the Revised Structures of Azaspiracid-2 and Azaspiracid-3. *Angewandte Chemie* 2006, 118(16):2671–2677.

178. Ofuji K, Satake M, McMahon T, James KJ, Naoki H, Oshima Y, Yasumoto T: Structures of azaspiracid analogs, azaspiracid-4 and azaspiracid-5, causative toxins of azaspiracid poisoning in Europe. *Biosci Biotechnol Biochem* 2001, 65(3):740-742.

179. James KJ, Moroney C, Roden C, Satake M, Yasumoto T, Lehane M, Furey A: Ubiquitous 'benign' alga emerges as the cause of shellfish contamination responsible for the human toxic syndrome, azaspiracid poisoning. *Toxicon* 2003, 41(2):145-151.

180. James KJ, Furey A, Lehane M, Ramstad H, Aune T, Hovgaard P, Morris S, Higman W, Satake M, Yasumoto T: First evidence of an extensive northern European distribution of azaspiracid poisoning (AZP) toxins in shellfish. *Toxicon* 2002, 40(7):909-915.

181. Hess P, Nguyen L, Aasen J, Keogh M, Kilcoyne J, McCarron P, Aune T: Tissue distribution, effects of cooking and parameters affecting the extraction of azaspiracids from mussels, *Mytilus edulis*, prior to analysis by liquid chromatography coupled to mass spectrometry. *Toxicon* 2005, 46(1):62-71.

182. Torgersen T, Bremnes NB, Rundberget T, Aune T: Structural confirmation and occurrence of azaspiracids in Scandinavian brown crabs (*Cancer pagurus*). *Toxicon* 2008, 51(1):93-101.

183. Magdalena AB, Lehane M, Moroney C, Furey A, James KJ: Food safety implications of the distribution of azaspiracids in the tissue compartments of scallops (*Pecten maximus*). *Food Addit Contam* 2003, 20(2):154-160.

184. Taleb H, Vale P, Amanhir R, Benhadouch A, Sagou R, Chafik A: First detection of azaspiracids in mussels in north west Africa. *Journal of Shellfish Research* 2006, 25(3):1067-1070.

185. James KJ, Furey A, Satake M, Yasumoto T: Azaspiracid poisoning (AZP): a new shellfish toxic syndrome in Europe. *Harmful Algal Blooms* 2000:250-253.

186. James KJ, Lehane M, Moroney C, Fernandez-Puente P, Satake M, Yasumoto T, Furey A: Azaspiracid shellfish poisoning: unusual toxin dynamics in shellfish and the increased risk of acute human intoxications. *Food Addit Contam* 2002, 19(6):555-561.

187. Alfonso A, Vieytes MR, Ofuji K, Satake M, Nicolaou KC, Frederick MO, Botana LM: Azaspiracids modulate intracellular pH levels in human lymphocytesstar. *Biochemical and Biophysical Research Communications* 2006, 346(3):1091-1099

188. Vilarino N, Nicolaou KC, Frederick MO, Cagide E, Ares IR, Louzao MC, Vieytes MR, Botana LM: Cell growth inhibition and actin cytoskeleton disorganization induced by azaspiracid-1 structure-activity studies. *Chem Res Toxicol* 2006, 19(11):1459-1466.

189. Vale C, Nicolaou KC, Frederick MO, Gomez-Limia B, Alfonso A, Vieytes MR, Botana LM: Effects of azaspiracid-1, a potent cytotoxic agent, on primary neuronal cultures. A structure-activity relationship study. *J Med Chem* 2007, 50(2):356-363.

190. Vale C, Wandscheer C, Nicolaou KC, Frederick MO, Alfonso C, Vieytes MR, Botana LM: Cytotoxic effect of azaspiracid-2 and azaspiracid-2-methyl ester in cultured neurons: involvement of the c-Jun N-terminal kinase. *J Neurosci Res* 2008, 86(13):2952-2962.

191. EFSA: Marine biotoxins in shellfish – Azaspiracid group. Scientific Opinion of the Panel on Contaminants in the Food chain. *European Food Safety Authority Journal* 2008, 723.

192. Alfonso C, Alfonso A, Otero P, Rodríguez P, Vieytes MR, Elliot C, Higgins C, Botana LM: Purification of five azaspiracids from mussel samples contaminated with DSP toxins and azaspiracids. *Journal of Chromatography B* 2008, 865:133-140.

193. These A, Scholz J, Preiss-Weigert A: Sensitive method for the determination of lipophilic marine biotoxins in extracts of mussels and processed shellfish by high-performance liquid chromatography-tandem mass spectrometry based on enrichment by solid-phase extraction. *J Chromatogr A* 2009, 1216(21):4529-4538.

194. Gerssen A, McElhinney MA, Mulder PP, Bire R, Hess P, de Boer J: Solid phase extraction for removal of matrix effects in lipophilic marine toxin analysis by liquid chromatography-tandem mass spectrometry. *Anal Bioanal Chem* 2009, 394(4):1213-1226.

195. Duagbjerg N, Hansen G, Larsen J, Moestrup O: Phylogeny of Some of the Major Genera of Dinoflagellates Based on Ultrastructure and Partial LSU rDNA

Sequence Data, Including the Erection of Three New Genera of Unarmored Dinoflagellates. *Phycologia* 2001, 39:302-317.

196. Brand LE, Compton A: Long Term Increase in *Karenia brevis* Abundance Along the Southwest Florida Coast. . *Harmful Algae* 2007, 6:232-252.
197. Bourdelais AJ, Tomas CR, Naar J, Kubanek J, D.G. B: New Fish Killing Alga in Coastal Delaware Produces Neurotoxins. . *Env Health Perspect* 2002, 110:465-470.

198. Gunter G, Williams RH, Davis CC, Walton Smith GF: Catastrophic Mass Mortality of Marine Animals and Coincident Phytoplankton Bloom on the West Coast of Florida, November 1946 to August 1947. *Ecological Monographs* 1948, 18:309-324.

199. O'Shea TJ, Rathbun GB, Bonde RK, Buergelt CD, Odell DK: An Epizootic of Florida Manatees Associated with a dinoflagellate bloom. *Marine Mammal Science* 1991, 7(2):165-179.

200. Lin Y, Risk M, Ray SM, Van Engen D, Clardy J, Golik J, James JC, Nakanishi K: Isolation and structure of brevetoxin B from the "red tide" dinoflagellate *Ptychodiscus brevis* (*Gymnodinium breve*). *J Am Chem Soc* 1981, 103(22):6773-6775.

201. Shimizu Y, Chou H-N, Bando H, Van Duyne G, Clardy J: Structure of brevetoxin A (GB-1 toxin), the most potent toxin in the Florida red tide organism *Gymnodinium breve* (*Ptychodiscus brevis*). *J Am Chem Soc* 1986, 108(3):514-515.

202. Ramsdell JS: The Molecular and Integrative Basis to Brevetoxin Toxicity. In *Seafood and Freshwater Toxins: Pharmacology, Physiology, and Detection, 2nd edition*. Edited by Botana LM. Boca Raton, FL: Taylor and Francis Group; 2008:519-545.

203. Dechraoui MY, Naar J, Pauillac S, Legrand AM: Ciguatoxins and Brevetoxins, Neurotoxic Polyether Compounds Active on Sodium Channels. *Toxicon* 1999, 37:125-143.

204. Bottein Dechraoui M-Y, Tiedeken JA, Persad R, Wang Z, Granade HR, Dickey RW, Ramsdell JS: Use of Two Detection Methods to Discriminate Ciguatoxins from Brevetoxins: Application to Great Barracuda from Florida Keys. *Toxicon* 2005, 46:261-270.

205. Gessner BD, McLaughlin JB: Epidemiologic impact of toxic episodes: neurotoxic toxins. In *Seafood and Freshwater Toxins: Pharmacology, Physiology, and Detection, 2nd edition*. Second edition. Edited by Botana LM. Boca Raton, FL: Taylor and Francis. CRC Press; 2008:77-103.

206. Bossart GD, Baden DG, Ewing RY, Roberts B, Wright Sd: Brevetoxicosis in manatees (Trichechus manatus latirostris) from the 1996 epizootic: gross, histologic, and immunohistochemical features. *Toxicol Pathol* 1998, 26:276-282.

207. Kreuder C, Mazet JA, Bossart GD, Carpenter TE, Holyoak M, Elie MS, Wright SD: Clinicopathologic features of suspected brevetoxicosis in double-crested

cormorants (*Phalacrocorax auritus*) along the Florida Gulf Coast. *J Zoo Wildl Med* 2002, 33(1):8-15.

208. Flewelling LJ, Naar JP, Abbott JP, Baden DG, Barros NB, Bossart GD, Bottein MY, Hammond DG, Haubold EM, Heil CA *et al*: Brevetoxicosis: red tides and marine mammal mortalities. *Nature* 2005, 435:755-756.

209. Bean JA, Fleming LE, Kirkpatrick B, Backer LC, Nierenberg K, Reich A, Cheng YS, Wanner A, Benson J, Naar J *et al*: Florida Red Tide Toxins (Brevetoxins) and Longitudinal Respiratory Effects in Asthmatics. *Harmful Algae* 2011, 10(6):744-748.

210. Kirkpatrick B, Fleming LE, Bean JA, Nierenberg K, Backer LC, Cheng YS, Pierce R, Reich A, Naar J, Wanner A *et al*: Aerosolized Red Tide Toxins (Brevetoxins) and Asthma: Continued health effects after 1 hour beach exposure. *Harmful Algae* 2011, 10(2):138-143.

211. APHA: Method for Ptychodiscus brevis toxins. In *Laboratory procedures for the examination of seawater and shellfish, 5th ed*. Washington, D. C., USA: American Public Health Association; 1985:64-80.

212. Rodríguez-Velasco ML: Toxin Monitoring Programs and Regulatory View. In *Seafood and Freshwater Toxins: Pharmacology, Physiology, and Detection, 2nd edition*. Edited by Botana LM. Boca Raton, FL: Taylor and Francis Group; 2008:919-929.

213. Naar J, Bourdelais A, Tomas C, Kubanek J, Whitney PL, Flewelling L, Steidinger K, Lancaster J, Baden DG: A Competitive ELISA to Detect Brevetoxins from *Karenia brevis* (Formerly *Gymnodinium Breve*) in Seawater, Shellfish, and Mammalian Body Fluid. *Environ Health Persp* 2002, 110:179-185.

214. Poli MA, Rivera VR, Neal DD, Baden DG, Messer SA, Plakas SM, Dickey RW, El Said KR, Flewelling L, Green D *et al*: An electrochemiluminescence-based competitive displacement immunoassay for the type-2 brevetoxins in oyster extracts. *Journal of AOAC International* 2007, 90:173-178.

215. Twiner MJ, Bottein Dechraoui M-Y, Wang Z, Mikulski CM, Henry MS, Pierce RH, Doucette GJ: Extraction and Analysis of Lipophilic Brevetoxins from the Red Tide Dinoflagellate *Karenia brevis. Anal Biochem* 2007, 369:128-135.

216. Mouri R, Oishi T, Torikai K, Ujihara S, Matsumori N, Murata M, Oshima Y: Surface plasmon resonance-based detection of ladder-shaped polyethers by inhibition detection method. *Bioorg Med Chem Lett* 2009, 19(10):2824-2828.

217. Kreuzer MP, Pravda M, O'Sullivan CK, Guilbault GG: Novel electrochemical immunosensors for seafood toxin analysis. *Toxicon* 2002, 40(9):1267-1274.

218. Kulagina NV, Mikulski CM, Gray S, Ma W, Doucette GJ, Ramsdell JS, Pancrazio JJ: Detection of marine toxins, brevetoxin-3 and saxitoxin, in seawater using neuronal networks. *Environ Sci Technol* 2006, 40(2):578-583.

219. Hua Y, Lu W, Henry MS, Pierce RH, Cole RB: Online High Performance Liquid Chromatography-Electrospray Ionization Mass Spectrometry for the

Determination of Brevetoxins in 'Red Tide' Algae. *Anal Chem* 1995, 67:1815-1823.

220. Plakas SM, el-Said KR, Jester EL, Granade HR, Musser SM, Dickey RW: Confirmation of brevetoxin metabolism in the Eastern oyster (*Crassostrea virginica*) by controlled exposures to pure toxins and to *Karenia brevis* cultures. *Toxicon* 2002, 40(6):721-729.

221. Shea D: Analysis of brevetoxins by micellar electrokinetic capillary chromatography and laser-induced fluorescence detection. *Electrophoresis* 1997, 18:277-283.

222. Seki T, Satake M, Mackenzie L, Kaspar HF, Yasumoto T: Gymnodimine, a new marine toxin of unprecedented structure isolated from New Zealand oysters and the dinoflagellate, *Gymnodinium* sp. *Tetrahedron Letters* 1995, 36(39):7093-7096

223. Hu TM, Curtis JM, Oshima Y, Quilliam MA, Walter JA, Watsonwright WM, Wright JLC: Spirolide-B and spirolide-D, 2 novel macrocycles isolated from the digestive glands of shellfish. *J Chem Soc Chem Commun* 1995:2159-2161.

224. Uemura D, Chou T, Haino T, Nagatsu A, Fukuzawa S, Zheng S, Chen H: Pinnatoxin A: A Toxic Amphoteric Macrocycle from the Okinawan Bivalve *Pinna muricata*. *J Am Chem Soc* 1995, 117:1155-1156.

225. Takada N, Umemura N, Suenaga K, Uemura D: Structural determination of pteriatoxins A, B and C, extremely potent toxins from the bivalve *Pteria penguin*. *Tetrahedron Letters* 2001, 42(20):3495-3497.

226. Torigoe K, Murata M, Yasumoto T, Iwashita T: Prorocentrolide, a toxic nitrogenous macrocycle from a marine dinoflagellate, *Prorocentrum lima*. *J Am Chem Soc* 1988, 110:7876-7877.

227. Hu T, deFreitas ASW, Curtis JM, Oshima Y, Walter JA, Wright JLC: Isolation and Structure of Prorocentrolide B, a Fast-Acting Toxin from *Prorocentrum maculosum*. *J Nat Prod* 1996, 59(11):1010-1014.

228. Lu C-K, Lee G-H, Huang R, Chou H-N: Spiro-prorocentrimine, a novel macrocyclic lactone from a benthic *Prorocentrum* sp. of Taiwan. *Tetrahedron Letters* 2001, 42:1713-1716.

229. Zheng SZ, Huang FL, Chen SC, Tan XF, Zuo JB, Peng J, Xie RW: The isolation and bioactivities of pinnatoxin. *Chin J Mar Drugs* 1990, 33:33-35.

230. Miles C, Rundberget T, Sandvik M, Aasen JAB, Selwood AI: The presence of pinnatoxins in Norwegian mussels. *Report 07b of Veterinærinstituttets rapportserie* 2010.

231. Macpherson GR, Burton IW, LeBlanc P, Walter JA, Wright JL: Studies of the biosynthesis of DTX-5a and DTX-5b by the dinoflagellate *Prorocentrum maculosum*: regiospecificity of the putative Baeyer-Villigerase and insertion of a single amino acid in a polyketide chain. *J Org Chem* 2003, 68(5):1659-1664.

232. MacKinnon SL, Cembella AD, Burton IW, Lewis N, LeBlanc P, Walter JA: Biosynthesis of 13-desmethyl spirolide C by the dinoflagellate *Alexandrium ostenfeldii*. *J Org Chem* 2006, 71(23):8724-8731.

233. Aasen J, MacKinnon SL, LeBlanc P, Walter JA, Hovgaard P, Aune T, Quilliam MA: Detection and identification of spirolides in Norwegian shellfish and plankton. *Chem Res Toxicol* 2005, 18:509-515.

234. Ciminiello P, Dell'Aversano C, Fattorusso E, Magno S, Tartaglione L, Cangini M, Pompei M, Guerrini F, Boni L, Pistocchi R: Toxin profile of *Alexandrium ostenfeldii* (Dinophyceae) from the Northern Adriatic Sea revealed by liquid chromatography-mass spectrometry. *Toxicon* 2006, 47:597-604.

235. MacKinnon SL, Walter JA, Quilliam MA, Cembella AD, Leblanc P, Burton IW, Hardstaff WR, Lewis NI: Spirolides isolated from Danish strains of the toxigenic dinoflagellate *Alexandrium ostenfeldii*. *J Nat Prod* 2006, 69(7):983-987.

236. Amzil Z, Sibat M, Royer F, Masson N, Abadie E: Report on the first detection of pectenotoxin-2, spirolide-a and their derivatives in French shellfish. *Mar Drugs* 2007, 5(4):168-179.

237. Villar Gonzalez A, Rodriguez-Velasco ML, Ben-Gigirey B, Botana LM: First evidence of spirolides in Spanish shellfish. *Toxicon* 2006, 48(8):1068-1074.

238. Selwood A. In *6th International Conference on Molluscan Shellfish Safety*. Blenheim, New Zealand; 2007.

239. Jester R, Rhodes L, Beuzenberg V: Uptake of paralytic shellfish poisoning and spirolide toxins by paddle crabs (*Ovalipes catharus*) via a bivalve vector. *Harmful Algae* 2009, 8:369-376.

240. Cembella AD, Lewis NI, Quilliam MA: Spirolide composition of micro-extracted pooled cells isolated from natural plankton assemblages and from cultures of the dinoflagellate *Alexandrium ostenfeldii*. *Natural Toxins* 1999, 7(5):197-206.

241. Touzet N, Franco JM, Raine R: Morphogenetic diversity and biotoxin composition of *Alexandrium* (Dinophyceae) in Irish coastal waters. *Harmful Algae* 2008, 7(6):782-797.

242. Mountfort D, Beuzenberg V, MacKenzie L, Rhodes L: Enhancement of growth and gymnodimine production by the marine dinoflagellate, *Karenia selliformis*. *Harmful Algae* 2006, 5(6):658-664

243. Munday R, Towers NR, Mackenzie L, Beuzenberg V, Holland PT, Miles CO: Acute toxicity of gymnodimine to mice. *Toxicon* 2004, 44(2):173-178.

244. Kharrat R, Servent D, Girard E, Ouanounou G, Amar M, Marrouchi R, Benoit E, Molgo J: The marine phycotoxin gymnodimine targets muscular and neuronal nicotinic acetylcholine receptor subtypes with high affinity. *J Neurochem* 2008, 107(4):952-963.

245. Molgó J, Amar M, Aráoz R, Benoit E, Silveira P, Schlumberger S, Lecardeur S, Servent D: The dinoflagellate toxin 13-Desmethyl Spirolide-C broadly targets muscle and neuronal nicotinic acetylcholine receptors with high affinity. In *16th*

European Section Meeting of the International Society on Toxinolog. Leuven, Belgium, September 7-10; 2008.

246. Munday R: Toxicology of cyclic imines: gymnodimine, spirolides, pinnatoxins, pteriatoxins, prorocentrolide, spiro-prorocentrimine, and symbioimines. In *Seafood and Freshwater Toxins: Pharmacology, Physiology, and Detection, 2nd edition*. Edited by Botana LM. Boca Raton, FL: Taylor and Francis; 2008:581-594.
247. Otero P, Alfonso A, Rodriguez P, Rubiolo JA, Cifuentes JM, Bermudez R, Vieytes MR, Botana LM: Pharmacokinetic and toxicological data of spirolides after oral and intraperitoneal administration. *Food Chem Toxicol* 2011.

248. EFSA: Scientific Opinion on marine biotoxins in shellfish – Cyclic imines (spirolides, gymnodimines, pinnatoxins and pteriatoxins). Panel on Contaminants in the Food Chain (CONTAM). *European Food Safety Authority Journal* 2010, 8(6):1628.

249. Fux E, McMillan D, Bire R, Hess P: Development of an ultra-performance liquid chromatography-mass spectrometry method for the detection of lipophilic marine toxins. *J Chromatogr A* 2007, 1157(1-2):273-280.

250. Vilarino N, Fonfria ES, Molgo J, Araoz R, Botana LM: Detection of gymnodimine-A and 13-desmethyl C spirolide phycotoxins by fluorescence polarization. *Anal Chem* 2009, 81(7):2708-2714.

251. Fonfria ES, Vilarino N, Molgo J, Araoz R, Otero P, Espina B, Louzao MC, Alvarez M, Botana LM: Detection of 13,19-didesmethyl C spirolide by fluorescence polarization using Torpedo electrocyte membranes. *Anal Biochem* 2010, 403(1-2):102-107.

252. Otero P, Alfonso A, Alfonso C, Araoz R, Molgo J, Vieytes MR, Botana LM: First direct fluorescence polarization assay for the detection and quantification of spirolides in mussel samples. *Anal Chim Acta* 2011, 701(2):200-208.

253. Suárez-Isla BA: Paralytic Shellfish Toxins, Pharmacology, and Toxicology Biological Detection Methods. In *Seafood and Freshwater Toxins: Pharmacology, Physiology, and Detection, 2nd edition*. Edited by Botana LM. Boca Raton, FL: Taylor and Francis; 2008:197-205.

254. Anderson DM, Kulis DM, Doucette GJ, Gallagher JC, Balech E: Biogeography of toxic dinoflagellates in the genus *Alexandrium* from the northeastern United States and Canada. *Marine Biology* 1994, 120:467-478.

255. Mons MN, Van Egmond HP, Speijers GJA: Paralytic shellfish poisoning: A review.RIVM Report 388802 005. June 1998. 1998

256. Wang DZ: Neurotoxins from marine dinoflagellates: a brief review. *Mar Drugs* 2008, 6(2):349-371.

257. Kodama M: Paralytic Shellfish Poisoning Toxins: Biochemistry and Origin. *Aqua-BioScience Monographs* 2010, 3(1):1-38.

258. Deeds JR, Landsberg JH, Etheridge SM, Pitcher GC, Longan SW: Non-traditional vectors for paralytic shellfish poisoning. *Mar Drugs* 2008, 6(2):308-348.

259. Sato S, Kodama M, Ogata T, Saitanu K, Furuya M, Hirayama K, Kakinuma K: Saxitoxin a toxic principle of a freshwater puffer, *Tetraodon fangi*, in Thailand. *Toxicon* 1997, 35(1):137-140.

260. Etheridge SM: Paralytic shellfish poisoning: seafood safety and human health perspectives. *Toxicon* 2010, 56(2):108-122.

261. Negri AP, Jones GJ: Bioaccumulation of paralytic shellfish poisoning (PSP) toxins from the cyanobacterium *Anabaena circinalis* by the freshwater mussel *Alathyria condola*. *Toxicon* 1995, 33(5):667-678.

262. Alam M, Shimizu Y, Ikawa M, Sasner JJ: Reinvestigation of the toxins from the blue-green alga, *Aphanizomenon flos-aquae*, by a high performance chromatographic method. *Journal of Environmental Science and Health* 1978, 13(7):493-499.

263. Onodera H, Satake M, Oshima Y, Yasumoto T, Carmichael WW: New saxitoxin analogues from the freshwater filamentous cyanobacterium *Lyngbya wollei*. *Natural toxins* 1997, 5(146):146-151.

264. Carmichael WW, Evans WR, Yin QQ, Bell P, Moczydlowski E: Evidence for paralytic shellfish poisons in the freshwater cyanobacterium *Lyngbya wollei* (Farlow ex Gamont) comb. nov. *Applied and Environmental Microbiology* 1997, 63:3104-3110.

265. Wiese M, D'Agostino PM, Mihali TK, Moffitt MC, Neilan BA: Neurotoxic alkaloids: saxitoxin and its analogs. *Mar Drugs* 2010, 8(7):2185-2211.

266. Liu Y, Chen W, Li D, Shen Y, Li G: First report of aphantoxins in China-waterblooms of toxigenic Aphanizomenon flos-aquae in Lake Dianchi. *Ecotoxicol Environ Saf* 2006, 65(1):84-92.

267. Berry JP, Lind O: First evidence of "paralytic shellfish toxins" and cylindrospermopsin in a Mexican freshwater system, Lago Catemaco, and apparent bioaccumulation of the toxins in "tegogolo" snails (*Pomacea patula catemacensis*). *Toxicon* 2010, 55(5):930-938.

268. Ballot A, Fastner J, Wiedner C: Paralytic shellfish poisoning toxin-producing cyanobacterium *Aphanizomenon gracile* in northeast Germany. *Appl Environ Microbiol* 2010, 76(4):1173-1180.

269. Kodama M, Ogata T, Sakamoto S, Sato S, Honda T, Miwatani T: Production of paralytic shellfish toxins by a bacterium *Moraxella sp.* isolated from *Protogonyaulax tamarensis*. *Toxicon* 1990, 28:707-714.

270. Gallacher S, Flynn KJ, Franco JM, Brueggemann EE, Hines HB: Evidence for production of paralytic shellfish toxins by bacteria associated with *Alexandrium spp.* (Dinophyta) in culture. *Appl Environ Microbiol* 1997, 63:239-245.

271. Kodama M, Ogata T, Sato S, Sakamoto S: Possible association of marine bacteria with paralytic shellfish toxicity of bivalves. *Mar Ecol Prog Ser* 1990, 61:203-206.

272. Michaud S, Levasseur M, Doucette G, Cantin G: Particle size fractionation of paralytic shellfish toxins (PSTs): seasonal distribution and bacterial production in the St Lawrence estuary, Canada. *Toxicon* 2002, 40:1451-1462.

273. Gallacher S, Smith EA: Bacteria and paralytic shellfish toxins. *Protist* 1999, 150:245-255.

274. Shimizu Y: Chemistry and mechanism of action. In *Seafood and Freshwater Toxins: Pharmacology, Physicology and Detection*. Edited by Botana LM. New York, USA: Marcel Dekker; 2000:151–172.

275. Berlinck RGS, Kossuga MH: Natural guanidine derivatives. *Nat Prod Rep* 2005, 22:516-550.

276. Schantz EJ, Mold JD, Stanger DW, Shavel J, Bowden FJP, Lynch JM, Wyler RS, Riegel BR, Sommer H: Paralytic shellfish poison VI. A procedure for the isolation and purification of the poison from toxic clams and mussel tissues. *J Am Chem Soc* 1957, 79:5230-5235.

277. Schantz EJ, Chazarossian VE, Schnoes HK, Springer JP, Pezzanite JO, Claudy J: The structure of saxitoxin. *J Am Chem Soc* 1975, 97:1238-1239.

278. Bower DJ, Hart RJ, Matthews PA, Howden MEH: Nonprotein neurotoxins. *Clin Toxic* 1981, 18:813.

279. Dell'Aversano C, Walter JA, Burton IW, Stirling DJ, Fattorusso E, Quilliam MA: Isolation and Structure Elucidation of New and Unusual Saxitoxin Analogues from Mussels. *Journal of Natural Products* 2008, 71:1518-1523.

280. Llewellyn LE: Saxitoxin, a toxic marine natural product that targets a multitude of receptors. *Nat Prod Rep* 2006, 23:200-222.

281. Cestele S, Catterall WA: Molecular mechanisms of neurotoxin action on voltage-gated sodium channels. *Biochimie* 2000, 82(9-10):883-892.

282. Noda M, Suzuki H, Numa S, Stuhmer W: A single point mutation confers tetrodotoxin and saxitoxin insensitivity on the sodium channel II. *FEBS Lett* 1989, 259(1):213-216.

283. Catterall WA: Neurotoxins that act on voltage-sensitive sodium channels in excitable membranes. *Annu Rev Pharmacol Toxicol* 1980, 20:15-43.

284. Kao CY: Tetrodotoxin, saxitoxin and their significance in the study of excitation phenomena. *Pharmacol Rev* 1966, 18(2):997-1049.

285. Aune T: Risk Assessment of Marine Toxins. In *Seafood and Freshwater Toxins: Pharmacology, Physiology, and Detection, 2nd edition*. Edited by Botana LM. Boca Raton, FL: Taylor and Francis Group; 2008:3-17.

286. AOAC: Official method 959.08. Paralytic shellfish poison: biological method. First action, 1959. Final action, Sec. 49.10.01. In *Official methods of analysis, 18th edn*. Edited by Horwitz W. Gaithersburg, MD, USA.: AOAC International; 2005.

287. Lawrence JF, Niedzwiadek B, Menard C: Quantitative determination of paralytic shellfish poisoning toxins in shellfish using prechromatographic oxidation and liquid chromatography with fluorescence detection: Collaborative study. *J AOAC Int* 2005, 88(6):1714-1732.

288. AOAC: Paralytic shellfish poisoning toxins in shellfish, prechromatographic oxidation and liquid chromatography with fluorescence detection, fisrt action 2005, method 2005.06. In *Methods of Analysis of the Association of Official Analytical Chemists*. Gaithersburg, MD: AOAC International; 2006:83.

289. Sommer H, Meyer KF: Paralytic Shellfish Poisoning. *Archives of Pathology* 1937, 24:560-598.

290. Hollingworth T, Wekell MM: Fish and other Marine Products 959.08. Paralytic shellfish poisoning. Biological Method, Final Action. . In *Official Methods of Analysis of the Association of Official Analytical Chemists (15th Edition)*. Edited by Hellrich K. Arlington, Virginia, USA.; 1990:881-882.

291. Schantz EJ, McFarren EF, M.L. S, Lewis KH: Purified shellfish poison for bioassay standardization. *J Assoc Off Anal Chem* 1958, 41:160-168.

292. Cembella AD: In vitro biochemical methods and mammalian bioassays for phycotoxins. In *Manual of Harmful Marine Microalgae. Volume 33*. Edited by Hallegraeff GM, Anderson DM, Cembella AD. Paris, France: IOC Manual and Guides; 1995:177.

293. McCulloch AW, Boyd RK, de Freitas AS, Foxall RA, Jamieson WD, Laycock MV, Quilliam MA, Wright JL, Boyko VJ, McLaren JW: Zinc from oyster tissue as causative factor in mouse deaths in official bioassay for paralytic shellfish poison. *J Assoc Off Anal Chem* 1989, 72(2):384-386.

294. Sauer UG: Animal vs. non-animal tests for the monitoring of marine biotoxins in the EU. *Altex* 2005, 22(1):19-24.

295. Chu FS, Fan TSL: Indirect enzyme-linked immunosorbent assay for saxitoxin in shellfish. *J AOAC* 1985, 68:13-16.

296. Kawatsu K, Hamano Y, Sugiyama A, Hashizume K, Noguchi T: Development and Application of an Enzyme Immunoassay Based on a Monoclonal Antibody against Gonyautoxin Components of Paralytic Shellfish Poisoning Toxins. *J Food Protection* 2002, 65(8):1304-1308.

297. Mackintosh FH, Gallacher S, Shanks AM, Smith EA: Assessment of MIST Alert, a commercial qualitative assay for detection of paralytic shellfish poisoning toxins in bivalve molluscs. *J AOAC Int* 2002, 85(3):632-641.

298. Vieytes MR, Cabado AG, Alfonso A, Louzao MC, Botana AM, Botana LM: Solid-Phase Radioreceptor Assay for Paralytic Shellfish Toxins *Analytical Biochemistry* 1993, 211(1):87-93.

299. Ruberu SR, Liu YG, Wong CT, Perera SK, Langlois GW, Doucette GJ, Powell CL: Receptor binding assay for paralytic shellfish poisoning toxins: optimization and interlaboratory comparison. *J AOAC Int* 2003, 86(4):737-745.

300. Fonfría ES, Vilariño N, Campbell K, Elliott CT, Haughey SA, Ben-Gigirey B, Vieites JM, Kawatsu K, Botana LM: Paralytic Shellfish Poisoning Detection by Surface Plasmon Resonance-Based Biosensors in Shellfish Matrixes. *Anal Chem* 2007, 79:6303-6311.

301. Campbell K, Stewart LD, Doucette GJ, Fodey TL, Haughey SA, Vilarino N, Kawatsu K, Elliott CT: Assessment of specific binding proteins suitable for the detection of paralytic shellfish poisons using optical biosensor technology. *Anal Chem* 2007, 79(15):5906-5914.

302. Gawley RE, Mao H, Haque MM, Thorne JB, Pharr JS: Visible fluorescence chemosensor for saxitoxin. *J Org Chem* 2007, 72(6):2187-2191.

303. Cheun BS, Loughran M, Hayashi T, Nagashima Y, Watanabe E: Use of a channel biosensor for the assay of paralytic shellfish toxins. *Toxicon* 1998, 36(10):1371-1381.

304. Vélez P, Suárez-Isla BA, Sierralta J, Fonseca M, Loyola H, Johns DC, Tomaselli GF, Marbán E: Electrophysiological assay to quantify saxitoxins in contaminated shellfish. *Biophysical Journal* 1999, 76(1):A82-A82 Part 82.

305. Velez P, Sierralta J, Alcayaga C, Fonseca M, Loyola H, Johns DC, Tomaselli GF, Marban E, Suarez-Isla BA: A funcional assay for paralytic shellfish toxins that uses recombinant sodium channels. *Toxicon* 2001, 39:929-935.

306. Louzao MC, Vieytes MR, Baptista de Sousa JM, Leira F, Botana LM: A fluorimetric method based on changes in membrane potential for screening paralytic shellfish toxins in mussels. *Anal Biochem* 2001, 289(2):246-250.

307. Louzao M, Vieytes MR, Garcia Cabado A, Vieites Baptista De Sousa J, Botana L: A fluorimetric microplate assay for detection and quantitation of toxins causing paralytic shellfish poisoning. *Chem Res Toxicol* 2003, 16:433-438.

308. Thibault P, Pleasance S, Laycock MV: Analysis of paralytic shellfish poisons by capillary electrophoresis. *J Chromatogr* 1991, 542:483-501.

309. Sullivan J, Wekell MM, L. K: Application of HPLC for the determination of PSP toxins in shellfish. *J Food Sci* 1985, 50:26-29.

310. Oshima Y: Postcolumn derivatization liquid chromatographic method for paralytic shellfish toxins. *J AOAC Int* 1995, 78(2):528-532.

311. Alfonso A, Vieytes MR, Botana AM, Goenaga Xa, Botana LM: Preparation of mixtures of paralytic shellfish toxin (PSP) standards from mussel hepatopancreas. *Fresenius J Anal Chem* 1993(345):212-216.

312. Diener M, Erler K, Hiller S, Christian B, Luckas B: Determination of Paralytic Shellfish Poisoning (PSP) toxins in dietary supplements by application of a new HPLC/FD method. *European Food Research and Technology* 2006, 224(2):147-151.

313. Dell'Aversano C, Hess P, Quilliam MA: Hydrophilic interaction liquid chromatography-mass spectrometry for the analysis of paralytic shellfish poisoning (PSP) toxins. *Journal of Chromatography A* 2005, 1081(2):190-201.

314. Rourke WA, Murphy CJ, G. P, Van de Riet JM, Burns BG, Thomas KM, Quilliam MA: Rapid postcolumn methodology for determination of paralytic shellfish toxins in shellfish tissue. *Journal of AOAC International* 2008, 91(3):589-597.

315. Lawrence JF, Menard C: Liquid chromatographic determination of paralytic shellfish poisons in shellfish after prechromatographic oxidation. *J Assoc Off Anal Chem* 1991, 74(6):1006-1012.

316. Lawrence JF, Menard C, Cleroux C: Evaluation of prechromatographic oxidation for liquid chromatographic determination of paralytic shellfish poisons in shellfish. *J AOAC Int* 1995, 78(2):514-520.

317. Ben-Gigirey B, Rodríguez-Velasco ML, Villar-González A, Botana LM: Influence of the sample toxic profile on the suitability of a high performance liquid chromatography method for official paralytic shellfish toxins control. *J Chromatography A* 2007(1140):78-87.

318. Andrew DT, Deirdre MN, Robert GH, Steven M, Allan RR, Myriam A, David NL: Refinement and Extension of AOAC Method 2005.06 to Include Additional Toxins in Mussels: Single-Laboratory Validation. *Journal of AOAC International* 2009, 92(1).

319. Thomas K, Chung S, Ku J, Reeves K, Quilliam MA: *Molluscan Shellfish Safety*. In *The Marine Institute*. Edited by Henshilwood K, Deegan B, McMahon T, Cusack C, Keaveney S, Silke J, O'cinneide M, Lyons D, Hess P. Galway, Ireland; 2006:132-138.

320. Franco JM, Fernández-Vila P: Separation of Paralytic Shellfish Toxins by Reversed Phase High Performance Liquid Chromatography, with Postcolumn Reaction and Fluorimetric Detection. *Chromatographia* 1993, 35(9-12):613-620.

321. EFSA: Marine biotoxins in shellfish – Saxitoxin group. Scientific opinion of the Panel on Contaminants in the Food Chain. *European Food Safety Authority Journal* 2009, 1019:1-76.

322. Noguchi T, Arakawa O: Tetrodotoxin-distribution and accumulation in aquatic organisms, and cases of human intoxication. *Marine Drugs* 2008, 6(2):220-242.

323. Noguchi T, Ebesu JSM: Puffer poisoning: epidemiology and treatment. *J Toxicol, Toxin Rev* 2001, 20:1-10.

324. Hwang DF, Noguchi T: Tetrodotoxin poisoning. *Adv Food Nutr Res* 2007, 52:141-236.

325. Hwang PA, Tsai YH, Lin SJ, Hwang DF: The gastropod possessing TTX and/or PSP. *Food Reviews International* 2007, 23:321-340.

326. Hemmert CD: Tetraodon (pufferfish) poisoning. In *Seafood Safety*. Edited by Ahmed FE. Washington, DC: National Academy Press; 1974:100.

327. Nunez-Vazquez EJ, Yotsu-Yamashita M, Sierra-Beltran AP, Yasumoto T, Ochoa JL: Toxicities and distribution of tetrodotoxin in the tissues of puffer fish found in the coast of the Baja California Peninsula, Mexico. *Toxicon* 2000, 38(5):729-734.

328. Katikou P, Georgantelis D, Sinouris N, Petsi A, Fotaras T: First report on toxicity assessment of the Lessepsian migrant pufferfish *Lagocephalus sceleratus* (Gmelin, 1789) from European waters (Aegean Sea, Greece). *Toxicon* 2009, 54(1):50-55.

329. Golani D: Distribution of Lessepsian migrant fish in the Mediterranean. *Ital J Zool* 1998, 65:95-99.

330. Galil BS, Zenetos A: A sea change: exotics in the eastern Mediterranean Sea. . In *Invasive Aquatic Species of Europe: Distribution, Impacts and Management* Edited by Leppa¨koski E, Gollasch S, Olenin S. Dordrecht, The Netherlands: Kluwer Academic Publishers; 2002:325-336.

331. Eisenman A, Rusetski V, Sharivker D, Yona Z, Golani D: An odd pilgrim in the Holy Land. *The American Journal of Emergency Medicine* 2008, 26(3):383.

332. Bentur Y, Ashkar J, Lurie Y, Levy Y, Azzam ZS, Litmanovich L, Golik M, Gurevych B, Golani D, Eisenman A: Lessepsian migration and tetrodotoxin poisoning due to *Lagocephalus sceleratus* in the eastern Mediterranean. *Toxicon* 2008, 52:964-968.

333. Mosher HS, Fuhrman GJ, Fuhrman FA, Fischer HG: Tarichatoxin-tetrodotoxin, a potent neurotoxin. *Science* 1965, 144:1100-1110.

334. Miyazawa K, Noguchi T: Distribution and origin of tetrodotoxin. *J Toxicol, Toxin Rev* 2001, 20:11-33.

335. Sato S, Ogata T, Borja V, Gonzales C, Fukuyo Y, Kodama M: Frequent occurrence of paralytic shellfish poisoning toxins as dominant toxins in marine puffer from tropical water. *Toxicon* 2000, 38(8):1101-1109.

336. Carmichael WW, Mahmood NA: Toxins from freshwater cyanobacteria. *Seafood Toxins ACS Symp Ser* 1984, 262(32):377-389.

337. Hashimoto K, Noguchi T, Watabe S: New aspect of tetrodotoxin. In *Microbial Toxins in Foods and Feeds*. Edited by Pohland AE. New York: Plenum Press; 1990:159-172.

338. Narita H, Martsubara S, Miwa N, Akahane S, Murakami M, Goto T, Nara M, Noguchi T, Saito T, Shida Y *et al*: *Vibrio alginolyticus*, a TTX-producing bacterium isolated from the starfish *Astropecten polyacanthus*. *Bull Japan Soc Sci Fish* 1987, 47:935-941.

339. Noguchi T, Jeon JK, Arakawa O, Sugita H, Deguch Y, Shida Y, Hashimoto K: Occurrence of tetrodotoxin and anhydrotetrodotoxin in *Vibrio* sp. isolated from the intestine of a xanthid crab, *Atergatis floridus*. *J Biochem* 1986, 99:311-314.

340. Yasumoto T, Yasumura D, Yotsu M, Michishita T, Endo A, Kotaki Y: Bacterial production of tetrodotoxin and anhydrotetrodotoxin. *Agric Biol Chem* 1986, 50:793-795.

341. Simidu U, Noguchi T, Hwang DF, Shida Y, Hashimoto K: Marine bacteria which produce tetrodotoxin. *Appl Environ Microbiol* 1987, 53(7):1714-1715.

342. Arakawa O, Hwang D-F, Taniyama S, Takatani T: Toxins of Pufferfish That Cause Human Intoxications. In *Coastal Environmental and Ecosystem Issues of the East China Sea*. Edited by Ishimatsu A, Lie H-J: TERRAPUB and Nagasaki Universtiy; 2010:227-244.

343. Yokoo A: Chemical studies on pufferfish toxin (3) - separation of spheroidine. *Nippon Kagaku Zasshi* 1950, 71:590-592.

344. Tsuda K, Ikuma S, Kawamura M, Tachikawa R, Sakai K, Tamura C, Amakasu D: Tetrodotoxin. VII. On the structures of tetrodotoxin and its derivatives. *Chem Pharm Bull* 1964, 12:1357-1374.

345. Goto T, Kishi Y, Takahashi S, Hirata Y: Tetrodotoxin. *Tetrahedron* 1965, 21:2059-2088.

346. Nakamura M, Yasumoto T: Tetrodotoxin derivatives in puffer fish. *Toxicon* 1985, 23:271-276.

347. Yasumoto T, Yotsu M, Murate M, Naoki H: New tetrodotoxins analogues from the newt *Cynops ensicauda. J Am Chem Soc* 1988, 110:2344-2345.

348. Yotsu-Yamashita M, Schimmele B, Yasumoto T: Isolation and structural assignment of 5-deoxytetrodotoxin from the puffer fish *Fugu poecilonotus*. *Biosci Biotechnol Biochem* 1999, 63(5):961-963.

349. Jang JH, Yotsu-Yamashita M: 6,11-Dideoxytetrodotoxin from the puffer fish, *Fugu pardalis*. *Toxicon* 2007, 50:947-951.

350. Yotsu-Yamashita M, Yamagishi Y, Yasumoto T: 5,6,11-Trideoxytetrodotoxin from the puffer fish, *Fugu poecilonotus*. *Tetrahedron Lett* 1995, 36:9329-9332.

351. Khora SS, Yasumoto T: Isolation of 11-oxotetrodotoxin from the puffer, *Arthron nigropuntatus*. *Tetrahedron Letters* 1989, 30(33):4393-4394.

352. Endo A, Khora SS, Murata M, Yasumoto T: Isolation of 11-nor-tetrodotoxin-6(R)-ol and other tetrodotoxin derivatives from the puffer *Fugu niphobles. Tetrahedron Lett* 1988, 29:4127-4128.

353. Yotsu M, Hayashi Y, Khora S, Sato S, Yasumoto T: Isolation and structural assignment of 11-nortetrodotoxin-6(S)-ol from the puffer *Arothron nigropunctatus*. *Biosci Biotechnol Biochem* 1992, 56:370-371.

354. Yotsu-Yamashita M, Goto A, Nakagawa T: Identification of 4-S-cysteinyltetrodotoxin from the liver of the puffer fish, *Fugu pardalis*, and formation of thiol adducts of tetrodotoxin from 4,9-anhydrotetrodotoxin. *Chem Res Toxicol* 2005, 18:865-871.

355. Narahashi T, Moore JW, Poston RN: Tetrodotoxin derivatives: Chemical structure and blockage of nerve membrane conductance. *Science* 1967, 156:976-979.

356. Hille B: Ionic Channels of Excitable Membranes. *Sinauer Associates, Inc, Sunderland, MA* 1992.

357. Kao CY: Structure activity relations of tetrodotoxin, saxitoxin, and analogues. *Annals of the New York Academy of Sciences* 1986, 479:52-67.

358. Ochoa JL, Núñez EJ, Garcia A, Isabel Abdo I: Food Safety: Study about the toxicity of the cultivated bullseye puffer fish (*Sphoeroides annulatus*, Jenyns, 1843). *Panorama Acuícola Magazine* 2006.

359. Cohen NJ, Deeds JR, Wong ES, Hanner RH, Yancy HF, White KD, Thompson TM, Wahl M, Pham TD, Guichard FM *et al*: Public health response to puffer fish (Tetrodotoxin) poisoning from mislabeled product. *J Food Prot* 2009, 72(4):810-817.

360. Kawabata T: Assay method for tetrodotoxin. In *Food hygiene examination manual. Volume 2*. Tokyo: Japan Food Hygiene Association; 1978:232-240.

361. Raybould T, Bignami GS, Inouye LK, Simpson SB, Byrnes JB, Grothaus PG, Vann DC: A monoclonal antibody-based immunoassay for detecting tetrodotoxin in biological samples. *Journal of Clinical Laboratory Analysis* 1992, 6(2):65-72.

362. Kawatsu K, Hamano Y, Yoda T, Terano Y, Shibata T: Rapid and highly sensitive enzyme immunoassay for quantitative determination of tetrodotoxin. *Jpn J Med Sci Biol* 1997, 50(3):133-150.

363. Hamasaki K, Kogure K: A biological method for the quantitative measurement of tetrodotoxin (TTX): tissue culture bioassay in combination with a water-soluble tetrazolium salt. *Toxicon* 1996, 34(4):490-495.

364. Kreuzer MP, Pravda M, O'Sullivan CK, Guilbault GG: Novel electrochemical immunosensors for seafood toxin analysis. *Toxicon* 2002, 40:1267-1274.

365. Neagu D, Micheli L, Palleschi G: Study of a toxin-alkaline phosphatase conjugate for the development of an immunosensor for tetrodotoxin determination. *Anal BioAnal Chem* 2006, 385:1068-1074.

366. Yasumoto T, Michishita T: Fluorimetric determination of tetrodotoxin by high performance liquid chromatography. *Agric Biol Chem* 1985, 49:3077-3080.

367. Shoji Y, Yotsu-Yamashita M, Miyazawa T, Yasumoto T: Electrospray ionization mass spectrometry of tetrodotoxin and its analogs: liquid chromatography/mass spectrometry, tandem mass spectrometry, and liquid chromatography/tandem mass spectrometry. *Analytical Biochemistry* 2001, 290(1):10-17.

368. Yotsu-Yamashita M, Mebs D, Kwet A, Schneider M: Tetrodotoxin and its analogue 6-epitetrodotoxin in newts (*Triturus spp.*; *Urodela*, *Salamandridae*) from southern Germany. *Toxicon* 2007, 50(2):306-309.

369. Diener M, Christian B, Ahmed MS, Luckas B: Determination of tetrodotoxin and its analogs in the puffer fish *Takifugu oblongus* from Bangladesh by hydrophilic interaction chromatography and mass-spectrometric detection. *Anal Bioanal Chem* 2007, 389:1997-2002.

370. Jang JH, Lee JS, Yotsu-Yamashita M: LC/MS Analysis of Tetrodotoxin and Its Deoxy Analogs in the Marine Puffer Fish *Fugu niphobles* from the Southern Coast of Korea, and in the Brackishwater Puffer Fishes *Tetraodon nigroviridis*

and *Tetraodon biocellatus* from Southeast Asia. *Marine Drugs* 2010, 8:1049-1058.

371. Tsai YH, Hwang DF, Cheng CA, Hwang CC, Deng JF: Determination of tetrodotoxin in human urine and blood using C18 cartridge column, ultrafiltration and LC-MS. *Journal of Chromatography B* 2006, 832(1):75-80.

372. Jen HC, Lin SJ, Tsai YH, Chen CH, Lin ZC, Hwang DF: Tetrodotoxin poisoning evidenced by solid-phase extraction combining with liquid chromatography-tandem mass spectrometry. *Journal of Chromatography B* 2008, 871:95-100.

373. Busby P, Seamer C: New Zealand management programme: new approaches for the control and monitoring of phycotoxins. In *Proceedings of the Fourth International Conference on Molluscan Shellfish Safety, IOC UNESCO*. Edited by Villalba A, Reguera B, Romalde JL, Beiras R: Molluscan Shellfish Safety, Xunta de Galicia; 2003:153-158.

374. Commission CA: Joint FAO/WHO Food Standards Programme, Proposed Draft Standard for live bivalve molluscs and for raw bivalve molluscs processed for direct consumption or for further processing. In *Report of the Twenty-Eighth Session of the Codex Committee on Fish and Fishery Products (Beijing, China, 18-22 September, 2006), presented at Joint FAO/WHO Food Standards Programme, Codex Alimentarius Commission, 30th Session*. Rome, Italy, 2-7 July; 2007.

375. MacKenzie L, Beuzenberg V, Holland P, McNabb P, Selwood A: Solid phase adsorption toxin tracking (SPATT): a new monitoring tool that simulates the biotoxin contamination of filter feeding bivalves. *Toxicon* 2004, 44(8):901-918.

376. Pizarro G, Escalera L, González-Gil S, Franco JM, Reguera B: Growth, behaviour and cell toxin quota of *Dinophysis acuta* during a daily cycle. *Marine Ecology Progress Series* 2008, 353:89-105.

377. Lindahl O, Lundve B, Johansen M: Toxicity of *Dinophysis spp*. in relation to population density and environmental conditions on the Swedish west coast. *Harmful Algae* 2007, 6:218-231

378. Touzet N, Franco JM, Raine R: Characterization of Nontoxic and Toxin-Producing Strains of *Alexandrium minutum* (Dinophyceae) in Irish Coastal Waters. *Applied and Environmental Microbiology* 2007, 73(10):3333-3342.

379. Balech E: The genus *Alexandrium Halim* (Dinoflagellata). *Sherkin Island Marine Station, Sherkin Island Co, Cork, Ireland* 1995.

380. Shimizu Y, Yoshioka M: Transformation of paralytic shellfish toxins as demosntrated in scallop homogenates. *Science* 1981, 212:547-549.

381. Oshima Y: Chemical and enzymatic transformation of paralytic shellfish toxins in marine organisms. In *Harmful Marine Algal Blooms*. Edited by Lassus P, Arzul G, Erard E, Gentien P, Marcaillou C. Paris: Lavoisier; 1995:475-480.

382. Oshima Y, Sugino K, Itakura H, Hirota M, Yasumoto T: Comparative studies on paralytic shellfish toxin profile of dinoflagellates and bivalves. In *Toxic Marine Phytoplankton*. Edited by Graneli E, Sundstrom B, Edler L, Andersen DM. New York: Elsevier; 1990:391-396.

383. Jeon JK, Han MS, Lee HO: Paralytic shellfish toxins in the Mussel and *Alexandrium tamarense* (Dinoflagellate) from Gamraepo, Korea in 1989. In *Harmful and Toxic Algal Blooms*. Edited by Yasumoto T, Oshima Y, Fukuyo Y. Sendai: UNESCO; 1996:65-68.

384. Anderson DM: Bloom dynamics of toxic *Alexandrium* species in the northeastern U.S. *Limnol Oceanogr* 1997, 42:1009-1022.

385. Lilly EL, Halanych KM, Anderson DM: Phylogeny, biogeography, and species boundaries within the *Alexandrium minutum* group. *Harmful Algae* 2005, 4:1004-1020.

386. Penna A, Garces E, Vila M, Giacobbe MG, Fraga S, Luglie A, Bravo I, Bertozzini E, Vernesi C: *Alexandrium catenella* (Dinophyceae), a toxic ribotype expanding in the NW Mediterranean Sea. *Mar Biol* 2005, 148:13-23.

387. Touzet N, Keady E, Raine R, Maher M: Evaluation of taxa-specific real-time PCR, whole-cell FISH and morphotaxonomy analyses for the detection and quantification of the toxic microalgae *Alexandrium minutum* (Dinophyceae), Global Clade ribotype. *FEMS Microbiol Ecol* 2009, 67(2):329-341.

388. Galluzzi L, Penna A, Bertozzini E, Vila M, Garces E, Magnani M: Development of a real-time PCR assay for rapid detection and quantification of *Alexandrium minutum* (a Dinoflagellate). *Appl Environ Microbiol* 2004, 70(2):1199-1206.

389. SPIES-DETOX: Active biological monitoring and removal of toxins in aquaculture ecosystems and shellfish-including the development of a Solid-Phase In situ Ecosystem Sampler (SPIES) and detoxification of shellfish (DETOX). *European Commission Project under the sixth framework (2002-2006) Project No 0302790-2* 2008.

390. Van Dolah FM: Marine Algal Toxins: Origins, Health Effects, and Their Increased Occurrence. *Environ Health Perspect* 2000, 108:133-141.

391. Glibert PM, Anderson DM, Gentien P, Graneli E, Sellner KG: The global complex phenomena of harmful algae. *Oceanography* 2005, 18:136-147.

392. Haines A, Patz JA: Health effects of climate change. *JAMA* 2004, 291(1):99-103.

393. Patz JA, Campbell-Lendrum D, Holloway T, Foley JA: Impact of regional climate change on human health. *Nature* 2005, 438(7066):310-317.

394. Watson RT, Patz JA, Gubler DJ, Parson EA, Vincent JH: Environmental health implications of global climate change. *Journal of Environmental Monitoring* 2005, 7(9):834-843.

395. Maclean JL: Indo-Pacific Red Tides, 1985-1988. *Mar Pollut Bull* 1989, 20(7):304-310.

396. Moore SK, Trainer VL, Mantua NJ, Parker MS, Laws EA, Backer LC, Fleming LE: Impacts of climate variability and future climate change on harmful algal blooms and human health. *Environ Health* 2008, 7 Suppl 2:S4.

397. Tosteson TR: Caribbean ciguatera: A changing paradigm. *Revista de Biologia Tropical* 2004, 52:109-113.

398. Chateau-Degat ML, Chinain M, Cerf N, Gingras S, Hubert B, Dewailly E: Seawater temperature, *Gambierdiscus spp.* variability and incidence of ciguatera poisoning in French Polynesia. *Harmful Algae* 2005, 4(6):1053-1062.

399. Golani D: Colonization of the Mediterranean by Red Sea fishes via the Suez Canal - Lessepsian migration. In *Fish invasions of the Mediterranean Sea: Change and renewal* Edited by Golani D. A-G B. Sofia-Moscow: Pensoft Publishers; 2010:145-188.

400. Mosher HS: The chemistry of tetrodotoxin. *Annals of the New York Academy of Sciences* 1986, 479:32-43.

401. Ho PH, Tsai YH, Hwang CC, Hwang PA, Hwang JH, Hwang DF: Paralytic toxins in four species of coral reef crabs from Kenting National Park in southern Taiwan. *Food Control* 2006, 17:439-445.

402. Ngy L, Yu C-F, Taniyama S, Takatani T, Arakawa O: Co-occurrence of tetrodotoxin and saxitoxin in Cambodian marine pufferfish *Takifugu oblongus*. *African Journal of Marine Science* 2009, 31(3):349-354.

403. Lehane L: Paralytic shellfish poisoning: a potential public health problem. *Med J Aust* 2001, 175:343- 344.

404. O´Leary MA, Schneider JJ, Isbister GK: Use of high performance liquid chromatography to measure tetrodotoxin in serum and urine of poisoned patients. *Toxicon* 2004, 44:549-553.

405. Huang H-N, Lin J, Lin H-L: Identification and quantification of tetrodotoxin in the marine gastropod *Nassarius* by LC-MS. *Toxicon* 2008, 51:774-779.

406. Yotsu-Yamashita M, Sugimoto A, Takai A, Yasumoto T: Effects of specific modifications of several hydroxyls of tetrodotoxin on its affinity to rat brain membrane. *J Pharmacol Exp Ther* 1999, 289(3):1688-1696.

407. Chen CY, Chou HN: A modified high-performance liquid chromatography method for analysis of PSP toxins in dinoflagellate, *Alexandrium minutum*, and shellfish from Taiwan. *Food Research International* 2002, 35:715-720.

408. Vrana B, Mills GA, Allan IJ, Dominiak E, Svensson K, Knutsson J, Morrison G, Greenwood R: Passive sampling techniques for monitoring pollutants in water. *Trends in Analytical Chemistry* 2005, 24(10).

409. Fux E, Bire R, Hess P: Comparative accumulation and composition of lipophilic marine biotoxins in passive samplers and in mussels (*M. edulis*) on the West Coast of Ireland. *Harmful Algae* 2009, 8:523-537.

410. Rundberget T, Gustad E, Samdal IA, Sandvik M, Miles CO: A convenient and cost-effctive method for monitoring marine algal toxins with passive samplers. *Toxicon* 2009, 53:543-550.

411. Garnett CM, Rafuse CM, Lewis NI, Kirchhoff S, Cullen J, Quilliam MA: Monitroing of lipophilic shellfish toxins using SPATT (solid-phase adsorption

toxin tracking) in Nova Scotia, Canada. *Abstract O19-02 of the 12th International Conference on Harmful Algae; Copenhagen, Denmark, 4-8, September* 2006, 72.

412. Takahashi E, Yu Q, Eaglesham G, Connell DW, McBroom J, Costanzo S, Shaw GR: Occurrence and seasonal variations of algal toxins in water, phytoplankton and shellfish from North Stradbroke Island, Queensland, Australia. *Mar Env Res* 2007, 64:429-442.

413. Turrell E, Stobo L, Lacaze JP, Bresnan E, Gowland D: Development of an 'early warning system' for harmful algal blooms using solid-phase adsorption toxin tracking (SPATT). In *Oceans 2007 Europe International Conference: 2007; Aberdeen SCOTLAND* OCEANS 2007 - EUROPE; 2007:1304-1309

414. Turrell E, Stobo L, Lacaze JP: Optimization of Hydrophilic Interaction Liquid Chromatography/Mass Spectrometry and Development of Solid-Phase Extraction for the Determination of Paralytic Shellfish Poisoning Toxins. *Journal of AOAC International* 2008, 91(6).

415. Paredes I, Rietjens IM, Vieites JM, Cabado AG: Update of risk assessments of main marine biotoxins in the European Union. *Toxicon* 2011, 58(4):336-354.